UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

# INCLUSION OF *CISTUS LADANIFER* IN RUMINANT DIETS: AN APPROACH TO IMPROVE THE NUTRITIONAL VALUE OF EDIBLE FATS

OLINDA ROSA FRAGOSO DAS NEVES GUERREIRO

Orientadores: Professor Doutor Rui José Branquinho de Bessa

Doutora Eliana Alexandra Sousa Jerónimo

Doutora Maria de Fátima Pereira Duarte

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Produção Animal

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2018

Aos meus pais,

Sem vocês, não teria conseguido.
Obrigado por tudo

# ACKNOWLEDGEMENTS/AGRADECIMENTOS

Ao terminar uma das fases mais importantes da minha vida, não poderia deixar de agradecer a todos os que estiveram envolvidos no meu percurso enquanto estudante de doutoramento. Uma equipa que nos apoia, motiva e estimula a persistir mesmo nos momentos mais difíceis, é essencial para alcançar os nossos objetivos. Nesse aspeto tive imensa sorte pois os grupos de trabalho a que pertenço foram fundamentais para o meu sucesso enquanto investigadora. Assim, gostaria de agradecer:

À Faculdade de Medicina Veterinária da Universidade de Lisboa (FMV-UL) por ter possibilitado o desenvolvimento do meu projeto de doutoramento.

Ao Centro de Investigação Interdisciplinar em Sanidade Animal (CIISA), como instituição de acolhimento, onde me foram facultados todos os recursos humanos e materiais para a realização do trabalho.

Ao Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo (CEBAL) um agradecimento especial, pois para além de instituição de acolhimento, foi também onde me iniciei na investigação e que ao longo destes quase 10 anos tem disponibilizado todos os meios humanos e materiais para a realização do meu trabalho.

Ao Professor Doutor Rui Bessa, por ter aceitado ser meu orientador, por tudo o que me ensinou sobre bioidrogenação e ácidos gordos, pelos ensinamentos de análise estatística e delineamento experimental e pela sua disponibilidade e ajuda em todas as fases de execução deste trabalho.

À Doutora Eliana Jerónimo, minha co-orientadora, por todos os ensinamentos transmitidos, que foram para além dos científicos, pelo apoio, incentivo e conselhos que foram essenciais para a realização deste trabalho. Agradeço pela disponibilidade total, pela preciosa ajuda no planeamento dos ensaios, na elaboração e na redação dos artigos bem como desta tese, principalmente no último mês. Obrigado por acreditares sempre em mim e me motivares a fazer sempre mais e melhor. Ver o teu entusiasmo em querer saber sempre mais sobre a esteva, fez com que o meu entusiasmo e interesse pela esteva crescesse a cada dia. Obrigado por tudo.

À Doutora Fátima Duarte, pela co-orientação, pelo apoio e por ter acreditado em mim desde o início. Obrigado por me teres acompanhado ao longo dos últimos 10 anos, por me teres transmitido o gosto pela investigação e ciência, e por me incentivares a fazer sempre mais e melhor.

À Doutora Susana Alves pela ajuda com a análise e identificação dos ácidos gordos, com os ensaios *in vitro* e pelas sugestões durante a elaboração dos artigos, principalmente com o artigo dos ácidos gordos da esteva.

Ao Doutor Santos-Silva pela ajuda indispensável durante o ensaio, que decorreu nas instalações do Instituto Nacional de Investigação Agrária e Veterinária (INIAV), na Fonte Boa. Pela ajuda na interpretação dos resultados e acima de tudo pela hospitalidade com que me recebeu desde o primeiro dia na Fonte Boa.

À Eng. Teresa Dentinho, pela partilha de conhecimentos sobre os extratos de taninos de esteva e por toda a simpatia com que sempre me recebeu na Fonte Boa.

Ao Doutor João Almeida pela ajuda com a análise da qualidade da carne.

Ao Sr. José Batista pela pronta ajuda com as análises químicas e à D. Paula pela ajuda com os abates dos animais e processamento das carcaças.

Às colegas de doutoramento da FMV, Mónica e Alexandra pela ajuda durante os ensaios e pelo apoio ao longo destes anos.

A todos os colegas do CEBAL, que de alguma forma me apoiaram neste meu percurso, em especial ao Grupo de Compostos Bioactivos e ao Grupo de Valorização de Agro-Alimentos, sem esquecer todos aqueles que já passaram pelo CEBAL.

À Doutora Conceição Fernandes e à Doutora Ana Rita Prazeres por se preocuparem comigo e pelos incentivos para continuar.

A todos os colegas que me ajudaram nas recolhas de esteva, a vossa ajuda foi muito importante para a realização deste trabalho.

Ao David pela preciosa ajuda com a esteva, os taninos e por todas as outras coisas, e desculpa por estar sempre a pedir alguma coisa. À Liliana pela ajuda nas recolhas de esteva, pela companhia em Santarém, mesmo não estando a 100%, e com os lípidos, claro. Agradeço-vos pelo apoio, incentivo e amizade nesta última fase, foi mesmo "o *sprint* final". Obrigado por tudo.

À Daniela pelos momentos de apoio e alegria que em muito ajudaram a aliviar os dias.

À Teresa pelo apoio, incentivo e amizade e pela força nesta última fase.

Ao Miguel pela preciosa ajuda com as capas, e por estares sempre pronto a ajudar. Obrigado também pelos momentos de descontração e conversa.

Às minhas meninas Ana e Angela, a vossa amizade, apoio e incentivo foram essenciais para conseguir chegar ao fim em condições. Obrigado por tudo mesmo, pelas nossas longas conversas, por ouvirem todos os meus desabafos e problemas, e por me tentarem perceber, mesmo quando vos falava de bioidrogenação e afins. Sem vocês teria sido muito mais difícil, principalmente na parte final. O vosso "estás no bom caminho", "vais conseguir", "é possível" foi muito, mesmo muito importante e foi o que me deu forças no último mês, para conseguir terminar, sem me ir abaixo e sem estrar em stress. E claro que não poderia deixar de agradecer o enorme esforço e sacrifício que fizeram para me acompanharem quando eu precisava de chocolate. Obrigado por tudo mesmo!!

À minha família, pelo apoio ao longo desta caminhada. Em especial ao meu irmão Sérgio e à minha sobrinha Catarina, pelo carinho, amizade e incentivo e pelos momentos em que me distraiam e que tanto contribuíram para melhorar os meus dias. À Cristina pela amizade e apoio que significou muito para mim neste final de caminhada.

Por fim, um agradecimento muito especial aos meus pais, sem eles não teria conseguido chegar onde cheguei. Agradeço por todo o apoio, incentivo, conselhos e compreensão, mesmo nos dias mais stressantes e menos bons. Por todos os esforços que fizeram para eu ser o que sou hoje. OBRIGADO do fundo do coração por tudo, pois tudo o que sou devo a vocês.

Nem sempre foi fácil, mas consegui chegar ao fim.

A todos o meu mais sincero agradecimento.

This work was financed by Fundação para a Ciência e Tecnologia (FCT) through the individual fellowship SFRH/BD/84406/2012 and co-financed by the projects “Modulation of rumen biohydrogenation through polyunsaturated fatty acid overload and tannins” (PTDC/CVT/120122/2010) and “Funding of R&D units strategic plan - 2013/2015” (UID/CVT/00276/2013).

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

This work was also co-financed by Alentejo2020 program with the project “CistusRumen - Sustainable use of Rockrose (*Cistus ladanifer* L.) in small ruminants - Increase of the competitiveness and reduction of the environmental impact” (ALT20-03-0145-FEDER-000023) through Regional Development European Fund.

ALENTEJO 2020
PORTUGAL 2020
UNIÃO EUROPEIA
Fundo Europeu de Desenvolvimento Regional

# ABSTRACT

**Inclusion of *Cistus ladanifer* in ruminant diets: An approach to improve the nutritional value of edible fats**

*Cistus ladanifer* is a shrub quite abundant in Mediterranean countries, that when fed to lambs has been associated with changes in rumen biohydrogenation (BH), increasing *t*11-18:1 concentration in abomasal digesta and meat. The thesis main motivation was to further knowledge regarding *C. ladanifer* plant and to explore its utilization in nutritional strategies to ruminal BH modulation. In the first two experiments, seasonal variation of *C. ladanifer* aerial parts, with two ages, was characterized for chemical composition, including proximate composition and total phenols and condensed tannins (CT) contents, for *in vitro* digestibility, antioxidant activity and fatty acid (FA) profile. *Cistus ladanifer* was considered a nutritionally unbalanced feed, however, it may be used in ruminant nutrition, but only associated with other feeding resources to complement its nutritional imbalances. In third experiment, *C. ladanifer* fractions effects were tested on *in vitro* ruminal BH. Condensed tannins fraction was the most active on ruminal BH modulation, leading to a higher *t*11-18:1 accumulation and higher disappearance of substrate polyunsaturated FA. Therefore, we intended to determine which CT amount can optimize *t*11-18:1 ruminal synthesis. However, all CT fraction levels led to a depression of microbial growth without effects on ruminal BH. Last experiment was designed to explore effect of two levels of *C. ladanifer* CT (1.25% and 2.5%) and two ways of CT supply (*C. ladanifer* aerial parts and *C. ladanifer* CT extract) on lamb growth performance, carcass composition, meat quality and FA composition of lamb fat. The highest amount of *C. ladanifer* aerial part (2.5% of CT) had detrimental effects on growth performance, whereas, *C. ladanifer* CT extract inclusion in diet (1.25% of CT) led to the highest *t*11-18:1 increase in fat. So, we can conclude that *C. ladanifer* CT extract may be a good approach to improve the nutritional value of the ruminant edible fats.

**Keywords:** *Cistus ladanifer*, condensed tannins, biohydrogenation, fatty acids, ruminants

# RESUMO


**Inclusão de *Cistus ladanifer* na dieta de ruminantes: uma abordagem para melhorar o valor nutricional das gorduras edíveis**

As gorduras edíveis dos ruminantes têm sido associadas ao aumento do risco de doenças cardiovasculares, devido ao seu elevado conteúdo em ácidos gordos saturados, quantidades variáveis de ácidos gordos *trans* e baixo conteúdo de ácidos gordos polinsaturados. Este perfil de ácidos gordos (AG) resulta da bioidrogenação (BH) que os ácidos gordos polinsaturados da dieta sofrem no rúmen. Contudo, as gorduras edíveis dos ruminantes são naturalmente ricas em isómeros conjugados do ácido linoleico (CLA – conjugated linoleic acid), particularmente no isómero *c*9,*t*11-18:2, também designado por ácido ruménico. Diversos estudos *in vitro* e com modelos animais demonstraram que o *c*9,*t*11-18:2 possui atividades biológicas, como anti-inflamatória e anticarcinogénica. A manipulação da BH ruminal tem mostrado ser uma via para melhorar o valor nutricional das gorduras dos ruminantes, através do aumento da quantidade de ácidos gordos polinsaturados da dieta que escapam do rúmen sem sofrer alterações e do aumento da acumulação nos tecidos, e dos produtos dos intermediários da BH com efeitos benéficos, como CLA e do *t*11-18:1 (ácido vacénico), que é o principal precursor do *c*9,*t*11-18:2 nos tecidos. A inclusão de *Cistus ladanifer* L., um arbusto muito abundante na região Mediterrânea, em dietas à base de luzerna desidratada suplementada com 6% de óleos vegetais aumentou o *t*11-18:1 na digesta abomasal e na gordura intramuscular. *Cistus ladanifer* é rica em metabolitos secundários, que são compostos que não estão diretamente envolvidos nos processos de crescimentos, desenvolvimento e reprodução da planta, mas que contribuem para a adaptação e sobrevivência da planta. Contudo, a informação sobre a composição química da *C. ladanifer* é muito escassa, e não é claro qual a fração de *C. ladanifer* que possa ser responsável pela modulação da BH. A principal motivação desta tese foi aprofundar o conhecimento acerca da planta de *C. ladanifer*, e explorar a sua utilização em estratégias nutricionais para modular a BH ruminal. Nos dois primeiros ensaios, a variação sazonal das partes aéreas de *C. ladanifer*, com duas idades, foi caraterizada para a composição química, incluindo a composição proximal e o conteúdo em fenóis totais e taninos condensados (TC), para a digestibilidade *in vitro*, atividade antioxidante e perfil de AG. A parte aérea de *C. ladanifer* apresentou baixos teores de proteína, moderado conteúdo de constituintes parietais e baixa digestibilidade *in vitro*. Também foram encontrados na parte aérea da planta elevados teores em fenóis totais e TC, tendo estes aumentado durante o verão. A *Cistus ladanifer* é rica em AG saturados (73-82% do total de AG), e foram detetados pela primeira vez em arbustos

dois AG de cadeia ramificada (*iso*-19:0 e *iso*-21:0), cujo conteúdo aumentou no verão e outono. *Cistus ladanifer* foi considerada como um alimento nutricionalmente desequilibrado, com teores elevados de fenóis totais e de TC. Contudo, pode ser utilizado na nutrição de ruminantes, mas apenas associado com outros recursos alimentares que complementem os seus desequilíbrios nutricionais. No terceiro ensaio, o efeito de cinco frações de *C. ladanifer*, ricas em metabolitos secundários, foi testado sobre a BH ruminal *in vitro*. Apesar de várias frações de *C. ladanifer* terem induzido alterações no perfil de AG, a fração de TC foi a fração mais ativa na modulação da BH ruminal, levando a uma maior acumulação de *t*11-18:1 e maior desaparecimento dos ácidos gordos polinsaturados do substrato. Após estes resultados pretendeu-se determinar a quantidade de TC de *C. ladanifer* que pode otimizar a síntese ruminal de *t*11-18:1. Contudo, quando testámos níveis crescentes de TC (entre 1,5 e 6% de TC, correspondendo ao valor máximo a mesma dose usada no ensaio anterior), todos os níveis de TC levaram a uma diminuição dos AG ramificados e dos dimetil acetais o que sugere uma redução do crescimento microbiano, sendo o efeito sobre a BH limitado. A BH do *c*9,*c*12,*c*15-18:3 sofreu uma ligeira redução com o aumento dos níveis de TC, sem efeito na produção de *t*11-18:1. Face aos resultados obtidos nos ensaios anteriores, o último ensaio foi delineado para explorar o efeito de dois níveis de TC de *C. ladanifer* (1.25% e 2.5%) e duas formas de fornecimento (parte aérea de *C. ladanifer* e extrato de TC de *C. ladanifer*) no desempenho produtivo, composição da carcaça, qualidade da carne e composição de AG da gordura de borregos. A incorporação dos níveis mais elevados de TC (2.5%) levou a efeitos prejudiciais no desempenho produtivo dos animais, especialmente com a inclusão da parte aérea de *C. ladanifer*, sem efeitos benéficos na composição de AG da gordura intramuscular e subcutânea. A inclusão de extrato de TC de *C. ladanifer* (1.25% de TC) na dieta levou ao aumento de *t*11-18:1 na gordura intramuscular e subcutânea, contudo, não afetou a concentração de *c*9,*t*11-18:2. Com esta tese podemos concluir que o extrato de TC de *C. ladanifer* pode ser uma boa abordagem para melhorar o valor nutricional das gorduras edíveis dos ruminantes.

**Palavras-Chave**: *Cistus ladanifer*, taninos condensados, bioidrogenação, ácidos gordos, ruminantes

# LIST DE PUBLICATIONS

This PhD thesis was based on the following publications:

**Olinda Guerreiro**, Maria T. Dentinho, Olga C. Moreira, Ângela R. Guerra, Patrícia A. B. Ramos, Rui J. B. Bessa, Maria F. Duarte, Eliana Jerónimo (2016). Potential of *Cistus ladanifer* L. (rockrose)in small ruminant diets – effect of season and plant age on chemical composition, *in vitro* digestibility and antioxidant activity. *Grass and Forage Science*, 71, 437-447.

**Olinda Guerreiro**, Susana P. Alves, Maria F. Duarte, Rui J. B. Bessa, Eliana Jerónimo (2015). *Cistus ladanifer* L. shrub is rich in saturated and branched fatty acids and their concentration increases in the Mediterranean dry season. *Lipids*, 50, 493-501.

**Olinda Guerreiro**, Susana P. Alves, Mónica Costa, Ângelo Cabo, Maria F. Duarte, Eliana Jerónimo, Rui J. B. Bessa (2016). Effects of extracts obtained from *Cistus ladanifer* L. on *in vitro* rumen biohydrogenation. *Animal Feed Science and Technology*, 219, 304-312.

**Olinda Guerreiro**, Susana P. Alves, Mónica Costa, Maria F. Duarte, Eliana Jerónimo, Rui J. B. Bessa. Effects of increasing doses of condensed tannins extract from *Cistus ladanifer* L. on *in vitro* ruminal fermentation and biohydrogenation. (In advanced preparation to be submitted to *Animal Feed Science and Technology*).

**Olinda Guerreiro**, Susana P. Alves, David Soldado, Liliana Cachucho, Alexandra Francisco, José Santos-Silva, Rui J. B. Bessa, Eliana Jerónimo. Inclusion of aerial part and condensed tannins extract from Cistus ladanifer L. on lamb diet – Effect on growth performance, carcass and meat quality and fatty acid composition of intramuscular and subcutaneous fat. (In advanced preparation to be submitted to *Meat Science*).

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| | |
|---|---|
| % | Percentage |
| *a** | Redness (CIELAB colour dimension) |
| ADF | Acid detergent fibre |
| ADG | Average daily gain |
| ADL | Acid detergent lignin |
| ARA | Arachidonic acid (20:4*n*-6) |
| *b** | Yellowness (CIELAB colour dimension) |
| BCFA | Branched chain fatty acids |
| BH | Biohydrogenation |
| BHA | Butylated hydroxyanisole |
| BHP | Biohydrogenation products |
| BHS | Biohydrogenation substrates |
| BHT | Butylated hydroxytoluene |
| BI | Biohydrogenation intermediates |
| *c* | *cis* |
| ºC | Degree Celsius |
| *C** | Chroma – colour saturation |
| *C. ladanifer* | *Cistus ladanifer* L. |
| CLA | Conjugated linoleic acid |
| cm | centimeter |
| CoA | Coenzyme A |
| CP | Crude protein |
| CT | Condensed tannins |
| Da | Dalton |
| DE | Dichloromethane extract |
| DM | Dry matter |
| DMA | Dimethylacetals |
| DMI | Dry matter intake |
| DPA | Docosapentaenoic acid (22:5*n*-3) |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| E | Energy |
| EE | Ether extract |
| EFSA | European Food Safety Authorized |

| | |
|---|---|
| EO | Essential oil |
| EPA | Eicosapentaenoic acid (20:5*n*-3) |
| FA | Fatty acids |
| FAME | Fatty acid methyl esters |
| FAO | Food and Agriculture Organization of United Nations |
| g | gram |
| *g* | Relative centrifuge force |
| GC | Gas chromatography |
| GC-FID | Gas chromatography with flame ionization |
| GC-MS | Gas chromatography – mass spectrometry |
| GE | Gross energy |
| h | hour |
| *H** | Hue angle |
| IVOMD | *in vitro* organic matter digestibility |
| kg | kilogram |
| KKCF | Kidney knob channel fat |
| L | litre |
| *L** | Lightess (CIELAB colour dimension) |
| LAB | Liquid-associated bacteria |
| LC-PUFA | Long chain polyunsaturated fatty acids - ≥ 20 carbons |
| LDL | Low density lipoprotein |
| LL | *Longissimus lumborum* |
| LT | *Longissimus thoracis* |
| m | metre |
| M | Molar |
| m/z | mass-to-charge ratio |
| mg | miligram |
| min | Minute |
| MJ | Mega Joule |
| mL | mililitre |
| mm | milimetre |
| mM | Milimolar |
| MUFA | Monounsaturated fatty acids |
| n.d. | not determined |
| *n*-3 LC-PUFA | Sum of *n*-3 long chain fatty acids |

| | |
|---|---|
| *n*-3 PUFA | Polyunsaturated fatty acids of the *n*-3 serie |
| *n*-6 LC-PUFA | Sum of *n*-6 long chain fatty acids |
| *n*-6 PUFA | Polyunsaturated fatty acids of the *n*-6 serie |
| NDF | Neutral detergent fibre |
| NTP | Non-tannin phenols |
| OBCFA | Odd and branched chain fatty acids |
| OCFA | Odd chain fatty acids |
| *P* | Probability |
| PEG | Polyethylene glycol |
| pH | Potential of hydrogen |
| ppm | parts per million |
| PUFA | Polyunsaturated fatty acids |
| RT | Room temperature |
| s.d. | Standard deviation |
| SAB | Solid-associated bacteria |
| SAS | Statistical analysis system (Software package) |
| SCD | Stearoyl-CoA desaturase |
| SEM | Standard error of mean |
| SFA | Saturated fatty acids |
| *t* | *trans* |
| TAE | Tannic acid equivalents |
| TP | Total phenolics |
| TVFA | Total volatile fatty acids |
| µg | Microgram |
| µL | Microlitre |
| µm | Micrometre |
| UV | Ultraviolet |
| v | volume |
| VFA | Volatile fatty acids |
| vis | visible |
| w | Weight |
| wk | Week |

# INTRODUCTION

The ruminant edible products have been associated to an increased risk of cardiovascular diseases, due to their high content on saturated fatty acids (SFA) and *trans* fatty acids (FA), and low content in polyunsaturated fatty acids (PUFA). In the rumen, the dietary lipids are extensively hydrolysed and the unsaturated FA released are extensively biohydrogenated (Jenkins, Wallace, Moate, & Mosley, 2008), resulting in production of high amounts of SFA and *trans* FA (Bessa *et al.*, 2007). However, ruminant edible products may also hold health benefits arising from FA, such as conjugated linoleic acid isomers (CLA), which have gained interest because of their potent anti-inflammatory, immunomodulatory, anti-obese and anti-carcinogenic activity (Kuhnt, Degen, & Jahreis, 2016; Shokryzadan *et al.*, 2017). The ruminant fats are naturally rich in CLA isomers, particularly rumenic acid (*c*9,*t*11-18:2) (Schmid, Collomb, Sieber, & Bee, 2006), which is formed by ruminal biohydrogenation (BH) of 18:2*n*-6 (Jenkins *et al.*, 2008), and mainly by endogenous conversion of *t*11-18:1 (which result from ruminal BH of 18:2*n*-6 and 18:3*n*-3 (Jenkins *et al.*, 2008)) via Stearoyl-CoA desaturase (SCD) in tissues (Griinari *et al.*, 2000). A good approach to improve ruminant fat healthiness is via modulation of ruminal BH, reducing the SFA and increasing either the escape of dietary PUFA, and the outflow of beneficial biohydrogenation intermediates (BI) (i.e. *t*11-18:1) from rumen.

Several studies have showed an opportunity to improve the nutritional value of ruminant fat through diet changes. Plant secondary metabolites, are a diverse group of molecules involved in the survival of plants to their environment but are not part of the primary metabolism of cell growth and reproduction, such as phenolic compounds and condensed tannins (CT). Plant secondary compounds has been used in ruminant diets or incubated on *in vitro* studies to assess ruminal BH modulation (Vasta & Luciano, 2011). *Cistus ladanifer* L. (also named rockrose), a shrub quite abundant in the Mediterranean countries, was incorporated in oil supplemented diets for lambs and induced ruminal BH changes, with *t*11-18:1 increase and decrease of 18:0 contents in abomasal digesta, and increase of *c*9,*t*11-18:2 and *t*11-18:1 contents in intramuscular fat, without compromise the animal productive performance and meat sensory attributes (Jerónimo *et al.*, 2012; Jerónimo *et al.*, 2010). *Cistus ladanifer* contains several secondary metabolites, but the information regarding its chemical and nutritional composition is still very scarce. The effects upon BH might be due to the secondary compounds, but is not clear which *C. ladanifer* fraction might be responsible for BH modulation.

This thesis is devoted to characterize the chemical and nutritional composition, as well as *C. ladanifer* FA profile for further potential use on ruminant diets. The thesis also intends to

modulate the ruminal BH with the incorporation of *C. ladanifer* aerial part and its extracts in diets, aiming the increase of beneficial BI in rumen and potentiate the nutritional value of lipid fraction from ruminant edible fats. Globally, the present work aims to further knowledge regarding *C. ladanifer* plant, and explore this plant utilization in nutritional strategies to modulate ruminal BH.

This thesis is structured in 7 chapters. In **Chapter 1**, "Scientific background and objectives", *C. ladanifer* general considerations, and its chemical composition will be present, and the nutritional value for ruminant diets. The general FA composition of ruminant meat, FA nutritional value, the consumptions and dietary recommendations will be summarily addressed. Finally, the dietary strategies to modulate the ruminal BH, in order to improve the FA composition of ruminant products, will be reviewed. After this brief overview, the specific objectives of the present work will be described. **Chapters 2** to **6** are based on scientific manuscripts already published (3), or in advanced preparation for submission to publication (2).

Previous work of our group showed that *C. ladanifer* in oil supplemented diets may be a good approach to increasing BI in lamb meat (Jerónimo *et al.*, 2010). However, *C. ladanifer* nutritional composition is not yet well studied, therefore the effect of seasonal and plant age on chemical composition, *in vitro* digestibility and antioxidant activity were evaluated, and the results are present in **Chapter 2**. The detailed *C. ladanifer* FA composition was reported, for the first time, as presented in **Chapter 3**.

In order to explore which *C. ladanifer* fraction was responsible for ruminal BH effects, previously obtained, within **Chapter 4** are presented the results obtained from *in vitro* trial, where we tested five fractions of *C. ladanifer*, rich in different secondary compounds, to understand which one might modify the ruminal BH, increasing beneficial BI content, and decreasing BH of dietary PUFA. These results demonstrated that CT fraction was the fraction presenting the better effect upon *in vitro* ruminal BH.

After identification the *C. ladanifer* fraction, responsible for the ruminal BH modulation, and following the same objective, i.e. increase beneficial BI and PUFA, we intended to establish *C. ladanifer* CT levels, which might allow to improve *in vitro* ruminal BH without compromising the ruminal fermentation (**Chapter 5**).

In **Chapter 6** are present the results obtained from one trial where it was assessed if the incorporation of *C. ladanifer* plant, or CT extract from *C. ladanifer*, in lamb diet might modify the ruminal BH. The thesis explored the utilization of two levels of CT and two ways of incorporation (*C. ladanifer* aerial part and its CT extract) in oil supplemented diets to improve

the nutritional value of ruminant fat, increasing *t*11-18:1, *c*9,*t*11-18:2 and PUFA on tissues, without compromise the productive performance of animals and meat quality.

Finally, **Chapter 7** intends to summarize and discuss in an integrated manner the results obtained in each of the five previous chapters, the main conclusions and relevant perspectives for future research are also presented in this topic.

# CHAPTER 1

# SCIENTIFIC BACKGROUND AND OBJECTIVES

## 1. 1 General considerations about *Cistus ladanifer* L.

*Cistus ladanifer* L., also known as rockrose, "esteva" in Portuguese, is a resinous and fragrant evergreen shrub, native from Mediterranean region. This shrub is considered an opportunistic species as it quickly occupies acidic, uncultured and degraded soils, being the dominant shrub of burned and overgrazed areas, inhibit the growth of other plants, repopulate metal-polluted sites and as it is highly combustible, which can be the origin of forest fires (Chaves, Escudero, & Gutiérrez-Merino, 1997a; Martin-Pinto, Vaquerizo, Penalver, Olaizola, & Oria-de-Rueda, 2006; Santos, Abreu, & Magalhães, 2016). Only a small part of the plant is used by the perfumery industry, through the extraction of an oleoresin, called labdanum, which has a well-known characteristic and persistent amber aroma (Moyler & Clery, 1997), and the extraction of essential oil.

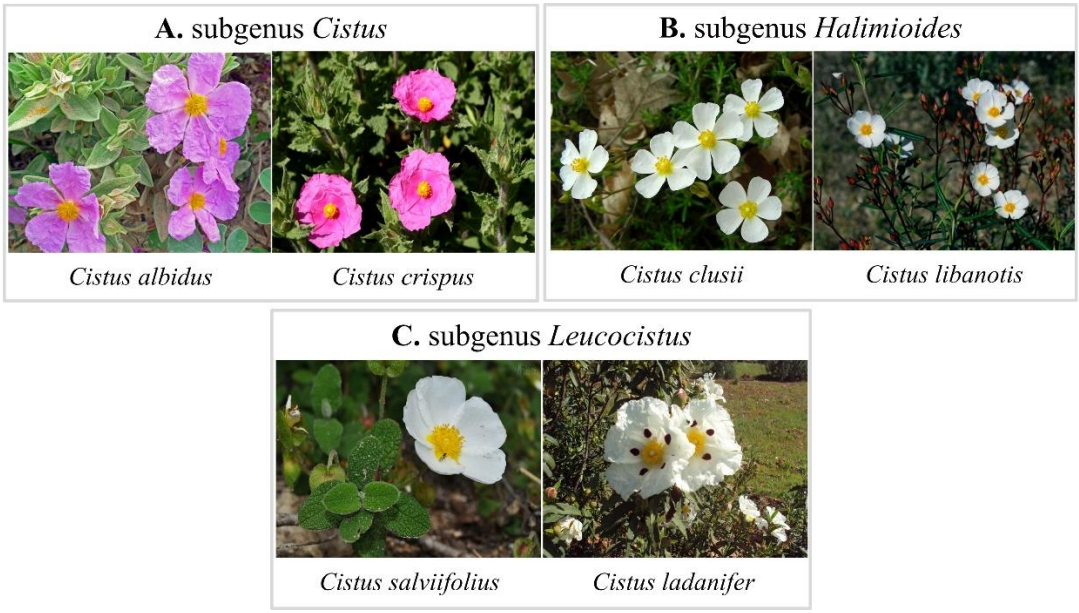
*Cistus ladanifer* is a rich source of secondary metabolites, a diverse group of molecules involved in the survival of plants to their environment but are not part of the primary biochemical pathways of cell growth and reproduction (Makkar, Siddhuraju, & Becker, 2007b). This shrub has been recognized as having diverse biological activities, which was mostly described on *in vitro* studies with essential oil and only a few studies describe the plant activities on *in vivo* animals, as antimicrobial (Ferreira *et al*., 2011; Greche *et al*., 2009; Morales-Soto *et al*., 2015; Tomas-Menor *et al*., 2013), antifungal (Barros *et al*., 2013), antihypertensive *(*Belmokhtar *et al*., 2009), anti-inflammatory and analgesic (El Youbi, El Mansouri, Boukhira, Daoudi, & Bousta, 2016) and antioxidant (Andrade, Gil, Breitenfeld, Domingues, & Duarte, 2009; Barrajon-Catalan *et al.*, 2010; Francisco *et al.*, 2015; Jerónimo *et al.*, 2012; Sanchez-Vioque *et al.*, 2013).

### 1.1.1 Taxonomy and phylogeny

The Cistaceae family comprise 8 genera and 180 species of shrubs, distributed in temperate and subtropical regions of the northern hemisphere. Within the Mediterranean region the Cistaceae family is the major shrub, being presented five, of the eight genera (*Cistus*, *Fumana*, *Halimium*, *Helianthemum* and *Tuberaria*) (Guzman & Vargas, 2009; Papaefthimiou *et al.*, 2014). Twenty one species of *Cistus* are recognized, spreading within white and pink-flowered lineages (Guzman & Vargas, 2009). The taxonomy and phylogenetic divisions are based on morphological characters, and plants specific genetic characteristics and chemical characterization (Barrajon-Catalan *et al*., 2011). *Cistus* genus is divided into three subgenus: *Cistus* (pink-flowered species) (Figure 1.1A), *Halimioides* (Figure 1.1B) and *Leucocistus*

(white-flowered species) (Figure 1.1C) (Guzman & Vargas, 2009). *Leucocistus* subgenus is the most numerous of the *Cistus* genus, and *C. ladanifer* belongs to this subgenus (Guzman & Vargas, 2005).

**Figure 1.1.** Three subgenus of *Cistus* genus and some examples of *Cistus* species belonging to each subgenus. A. subgenus *Cistus*; B. subgenus *Halimioides*; C. subgenus *Leucocistus*.




Pictures from http://www.floravascular.com and from personal collection (picture of *Cistus ladanifer*).

### 1.1.2 *Cistus ladanifer* morphology and distribution

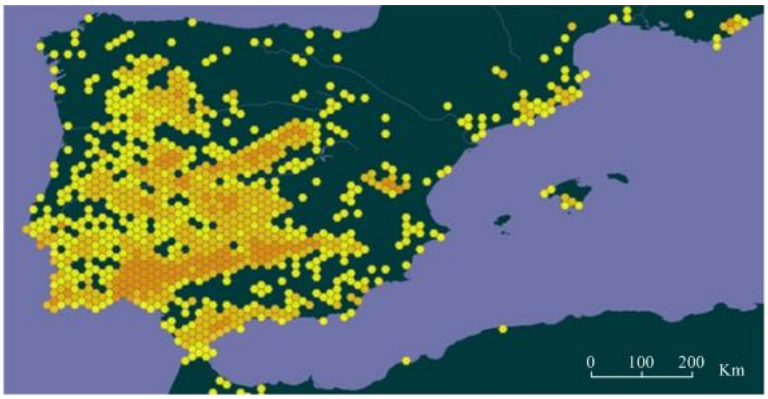
*Cistus ladanifer* (Figure 1.2) is a dicotyledonous perennial shrub, which can reach up to 2.5 m of height, with dense root and shoot systems, exhibiting branches of very rigid and lignified wood covered by a sticky and viscous bark (Frazão *et al.*, 2017). *Cistus ladanifer* presents full, opposite, leathery and sessile leaves, which are welded at the base, can grow up to 10 cm of length, with visible nerves (Figure 1.2B). The stems and leaves are covered by labdanum gum, produced from secretory trichomes. *Cistus ladanifer* plants present a large and terminal unique white flower, with a maroon blotch at the base of each petals (5 to 8 cm of diameter; Figure 1.2A) (Talavera, Gibbs, & Herrera, 1993). The seed heads of *C. ladanifer* are globular and lignified, with 6-12 valves. Each seed head produces a large number of seeds (aprox. 250 per valve), and total seed production by a single adult plant may be up to 158000 seeds each year (Figure 1.2C, D) (Bastida & Talavera, 2002; Demoly & Montserrat, 1993; Guzman & Vargas, 2009).

**Figure 1.2.** *Cistus ladanifer* L. morphology: A, flower buds and flower; B, young leaves; C, seed heads; D, open seed head with seeds.


Pictures were courtesy of David Soldado and Eliana Jerónimo

Natural habitats of *C. ladanifer* are located exclusively in the western Mediterranean area, from southern France to the north of Morocco and Algeria, and also in Balearic and Canarian islands (Figure 1.3), but principally in the southwestern region of Iberian Peninsula (Demoly & Montserrat, 1993). *Cistus ladanifer* grow in open areas of stony and infertile soils, generally in poor siliceous and acidic soils with shale, granite and sandstone origin. This species usually occupy areas of *Quercus* woodland that have been degraded by fire (Talavera *et al*., 1993).

**Figure 1.3.** *Cistus ladanifer* distribution area in southwestern Europe, using georeferenced data from Global Biodiversity Information Facility – *Cistus ladanifer* L.
In Global Biodiversity Information Facility Secretariat (2017). Global Biodiversity Information Facility Backbone Taxonomy. Checklist Dataset https://www.gbif.org/species/6437976



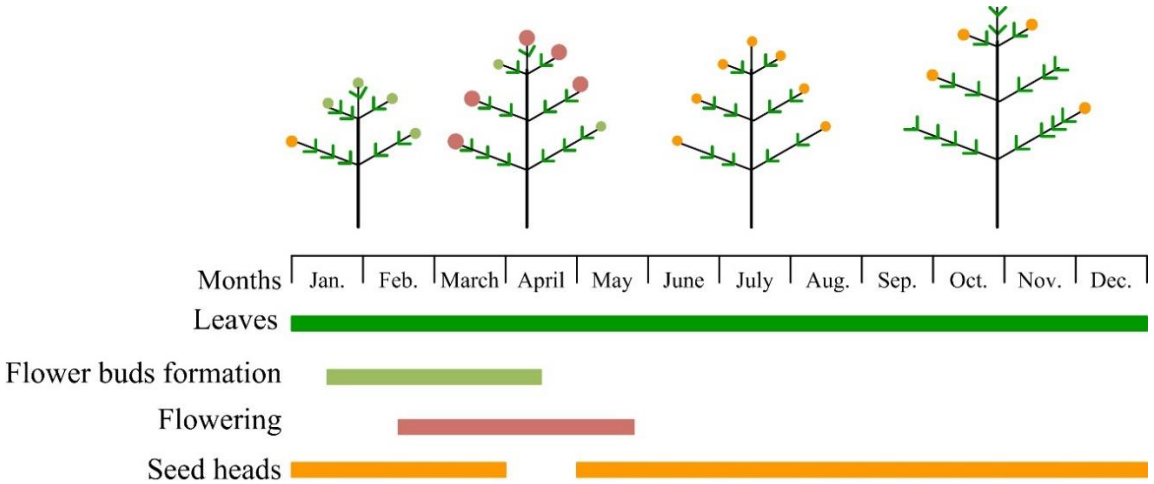
### 1.1.3 *Cistus ladanifer* phenology

The time and duration of the vegetative development of *C. ladanifer* (Figure 1.4) varies with the year and place of growth. Generally, it is considered that vegetative growth starts after the first autumn rains, when the new plants emerge and the new leaves regrowth in existing plants that developed in previous seasons, after being reduced during the summer dry season (Cabezudo, Navarro, Pérez Latorre, Nieto Caldera, & Orshan, 1992; Talavera *et al*., 1993).

The flower buds formation begins at the end of winter (lasts between January and May) (Figure 1.4), period coinciding with the greater abundance of water in the soil. A single bud is enclosed in 1-3 pairs of leaves that growth in the apex of each branch produced in the previous year (Figure 1.2B). A large number of flowers is produced during the spring, and the flowering phenophase extends from February to May, depending the location and the climate conditions (Talavera *et al*., 1993).

During seed heads maturation, leaves and bracts fall and the pedicels elongate and lignify. On early summer, the seed heads are mature and are exposed at the ends of the wood pedicels (Figure 1.2C). Flowering branch main axis continue to elongate and develop new leaves until the beginning of summer when a dormant period ensues. During summer seed heads begin to open (Figure 1.4) and, by early autumn, seed dispersal occurs following shaking of branches by wind and rain, being the seeds dispersed around the parent plants. The small seed size (Figure 1.2D) facilitate the accumulation and penetration on soils and leads to the formation of soil seed bank. The soil seed banks allow the escape from unfavorable conditions and guarantees propagation, after fire, for example (Frazão *et al*., 2017; Talavera *et al*., 1993). These seeds have a crusty and impermeable coat and germinate after thermal shock. *Cistus ladanifer* seeds have significant longevity as a result of the protection given by the impermeable coats. These ability to produce seeds with different degrees of dormancy is possibly a mechanism to adapt to new environmental scenarios (Gutterman, 1994). Leaf drop occurs all year, being more accentuated in summer (Cabezudo *et al*., 1992). At the end of summer, when the high temperature decrease and the nocturnal dew formation recommences, the plant axes reinitiate the vegetative growth (Talavera *et al*., 1993).

**Figure 1.4.** Phenology of *Cistus ladanifer*, and schematic representation of the branches in successive phenophases along the year.
Illustration of the leaves (dark green), flower buds formation (light green), flowering (pink) and seed heads formation and dispersal (yellow). Data from personal observation in field and adapted from Talavera *et al*. (1993) and Cabezudo *et al*. (1992).




### 1.1.3 Chemical and nutritional composition

There is a scarce information about *C. ladanifer* nutritional composition, and as far as we know, only one study described proximate and chemical composition of *C. ladanifer* (Dentinho, Navas, & Potes, 2005). On this study, the authors showed that the *C. ladanifer* presented a low crude protein (CP), moderate levels of parietal compounds, high levels of condensed tannins (CT) and low organic matter digestibility (Table 1.1) (Dentinho *et al.*, 2005).

**Table 1.1** Chemical and nutritional characterization of *Cistus ladanifer*

| **Parameters** | ***Cistus ladanifer*** |
|---|---|
| Dry matter (DM) (%) | 36.4 – 54.0 |
| Crude protein (% DM) | 7.7 – 8.8 |
| Ether extract (% DM) | 9.2 – 9.5 |
| Neutral detergent fibre (NDF) (% DM) | 31.8 – 32.0 |
| Acid detergent fibre (ADF) (% DM) | 23.2 – 23.6 |
| Acid detergent lignin (ADL) (% DM) | 7.0 – 8.2 |
| Organic matter digestibility (%) | 30.6 – 30.6 |
| Total phenols[1] | 7.6 – 11.6 |
| Condensed tannins[2] | n.d.– 10.0 |

[1] equivalents of tannic acid in % DM; [2] equivalents of catechin in % DM. n.d. not determined.
Values are the variations observed in two months, with three collections in successive years.
Adapted from Dentinho *et al*. (2005).

The chemical composition of *C. ladanifer* has been more studied, however, the great majority of the chemical characterization is focused on its essential oil (Gomes, Mata, & Rodrigues, 2005; Mariotti, Tomi, Casanova, Costa, & Bernardini, 1997; Vieira *et al.*, 2017) and its labdanum exudate (Alías, Sosa, Valares, Escudero, & Chaves, 2012; Chaves, Escudero, & Gutiérrez-merino, 1993; Chaves *et al.*, 1997a; Sosa, Alias, Escudero, & Chaves, 2005). More recently, other *C. ladanifer* extracts, obtained with several solvents, have been studied, but to a lesser extent (Andrade *et al*., 2009; Barrajon-Catalan *et al*., 2010).

Essential oils are mixture of natural secondary compounds obtained from the volatile fraction by steam distillation of aromatic plants. The most abundant compounds in essential oil are terpenes (mono- and sesquiterpenes) and can also contain phenol-derived aromatic compounds, characterized by low molecular weight (Gomes *et al.*, 2005; Vieira *et al.*, 2017). Labdanum exudate is obtained by boiling the plant material in water or by organic solvent extraction (Chaves *et al.*, 1993, 1997a; Greche *et al.*, 2009). Labdanum is mostly constituted by diterpenes and flavonoids, however, it also contains mono- and sesquiterpenes (Alías *et al*., 2012; Masa, Diaz, Gallego, & Lobon, 2016).

The most relevant volatile terpenes present on *C. ladanifer* essential oil and exudate extract, reported in the literature, are mono-, sesquiterpenes and norisoprenes (Table 1.2). Concerning the monoterpenes the major compounds identified are α-pinene and β-pinene (Gomes *et al.*, 2005; Mariotti *et al.*, 1997; Rincon, De Lucas, & Garcia, 2000; Teixeira, Mendes, Alves, & Santos, 2007; Vieira *et al.*, 2017), camphene (Zidane *et al*., 2013), and the major sesquiterpene is viridiflorol (Gomes *et al.*, 2005; Greche *et al.*, 2009). *Cistus ladanifer* also presents high proportions of the carotenoid derivative norisoprene called 2,2,6-trimethylcyclohexanone (Gomes *et al.*, 2005; Greche *et al.*, 2009; Mariotti *et al.*, 1997; Teixeira *et al.*, 2007; Zidane *et al.*, 2013), which is one of the main compound responsible for *C. ladanifer* odour (Ramalho, de Freitas, Macedo, Silva, & Silva, 1999).This compound was not found in other *Cistus* species. Diterpenes are rarely reported in *C. ladanifer* essential oil due to its low volatility. However, labdanum is rich in diterpenes mainly labdane-type ones (Alías *et al*., 2012; Greche *et al.*, 2009) as the labdanoic acid.

**Table 1.2.** Most relevant terpenes found in *Cistus ladanifer*

| Terpene class | Volatile terpenes | Variation (% of extract) |
|---|---|---|
| Monoterpenes | α-pinene | 0.8 – 50.4 |
| | β-pinene | 0.3 – 13.9 |
| | Camphene | 0.1 – 28 |
| | Limonene | 0.8 – 13.7 |
| | ρ-cymene | 0.2 – 11.1 |
| Sesquiterpenes | Viridiflorol | 0.3 – 5.9 |
| Norisoprenes | 2,2,6-trimethylcyclohexanone | 1.5 – 7.3 |

Variation corresponds to the minimum and maximum values obtained in several studies (Gomes *et al.*, 2005; Robles, Bousquet-Mélou, Garzino, & Bonin, 2003; Teixeira *et al.*, 2007; Viuda-Martos *et al.*, 2011)

Plant phenolics are involved in defense against ultraviolet radiation or aggression by pathogens, parasites and predators, and range from relatively simple molecules, as phenolic acids and flavonoids, to highly polymerized compounds, as lignins and tannins (Dai & Mumper, 2010). The *C. ladanifer* leaves and stems are rich in phenolic compounds, as ellagitannins (Barrajon-Catalan *et al*., 2011; Barrajon-Catalan *et al*., 2010; Barros *et al*., 2013), although, they were also described as containing high amounts of CT (Barrajon-Catalan *et al.*, 2010; Dentinho, Moreira, Pereira, & Bessa, 2007), but their chemical profile was not yet described for *C. ladanifer* plants. Flavonoids are another group abundantly present on *C. ladanifer* exudate, represented by apigenin, kaempferol and its methylated derivatives (Chaves *et al.*, 1997a; Sosa *et al.*, 2005). The exposure to high solar irradiance and hydric stress (drought), in summer, are the most documented stresses, which increase flavonoids, specially methylated ones, as well as labdanum production (Chaves *et al.*, 1993, 1997a; Sosa *et al.*, 2005).

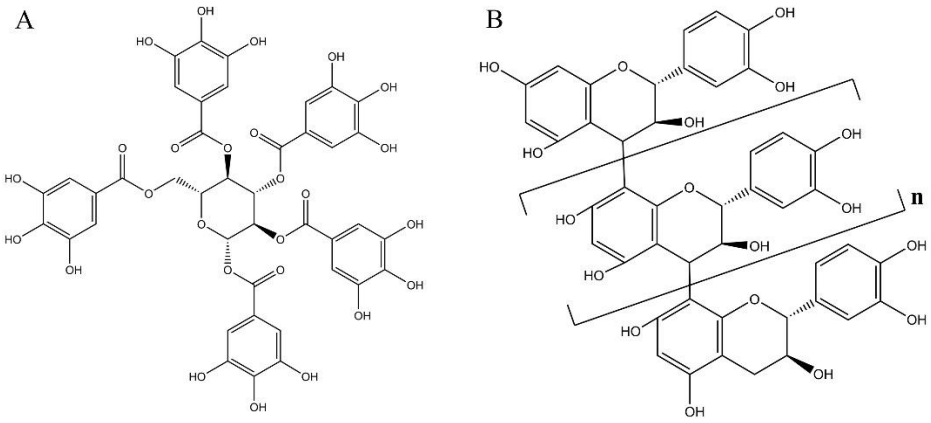
### 1.1.4 Utilization of *Cistus ladanifer* in ruminant diets

Shrubs are important as browse species in small ruminants feeding systems in Mediterranean areas, supplying energy and nutrients during pasture scarcity periods (Bruno-Soares, Matos, & Cadima, 2011). The rearing of small ruminants in Mediterranean area is limited by seasonal feed deficits, mostly during summer drought period, which may require expensive feed supplementation. The use of shrubs for partial cereal replacement in animal diets, mainly for small ruminants, has been explored in order to reduce the production costs of livestock feeding (Vasta & Luciano, 2011). Shrubs has been considered as alternative feed sources, and may have positive or negative effects, since they are a rich source of secondary compounds, which may be beneficial for animal production, or may limit their utilization as animal feed.

*Cistus ladanifer* is considered an unbalanced feed, with poor nutritional value, that can be considered as supplement and associated with other feeding resources that complements its nutritional imbalances, and also presents high content of antinutritional factors, as CT (Dentinho *et al.*, 2005).

Tannins are classified into hydrolysable and condensed tannins, based on the molecular structure (Figure 1.5). The hydrolysable tannins contain a carbohydrate (generally D-glucose) as a central core (Figure 1.5A). The hydroxyl groups of these carbohydrates are esterified with phenolic groups such as gallic or ellagic acid, whereas the CT, also known as proanthocyanidins, are complexes of oligomers and polymers of flavonoid units (flavan-3-ol and flavan-3,4-diols) linked by carbon-carbon bonds (Figure 1.5B) (Hagerman, Robbins, Weerasuriya, Wilson, & Mcarthur, 1992). Condensed tannins molecular weight range from 1000 to 20 000 Da (Hagerman, 1992).

**Figure 1.5.** Structures of hydrolysable (A) and condensed tannins (B)
A, hydrolysable tannins (example of pentagalloylglucose, gallic acid ester-linked to a hexose moiety); B, condensed tannins (polymers of flavan-3-ols). Adapted from Hagerman *et al*. (2002)




Condensed tannins are considered as antinutritional compounds since they form complexes with proteins, polysaccharides and minerals, conditioning its nutritive utilization (McSweeney, Palmer, Bunch, & Krause, 2001). Tannins are considered to have both adverse and beneficial effects, depending on chemical structure and concentration in diets, composition of basal diet, and other factors intrinsically associated to the animals, such as animal species and physiological stage (Makkar, Francis, & Becker, 2007a; Piluzza, Sulas, & Bullitta, 2014; Waghorn, 2008). Adverse effects include reduction of voluntary feed intake, digestibility of fibre and nitrogen, and animal performance (Min, Barry, Attwood, & McNabb, 2003; Waghorn, 2008). On the other hand, tannins may induce several beneficial effects, namely prevent bloat; improve protein utilization during digestion; reduce the methane emissions; act to control internal parasites and induce enhancements in growth performance, wool growth and milk production (Min *et al*., 2003; Piluzza *et al*., 2014; Waghorn, 2008). The negative effect on

voluntary feed intake could be explained by the reduction in palatability that could be due to a reaction between the tannins and the salivary mucoproteins, or through a direct reaction with the taste receptors, causing an astringent sensation (Frutos, Hervás, Giráldez, & Mantecón, 2004). However, ruminants which are exposed to tannins rich diets for a long time could develop diverse adaptation mechanisms to overcome deleterious effects of tannins. The ruminal adaptation to CT could be a defense mechanism, and some bacteria grown in the presence of CT secrete exo-polysaccharides forming a protective layer around the cells, therefore protecting cells from the CT action (Krause, Smith, Brooker, & McSweeney, 2005).

Several authors described beneficial and detrimental effects on animal production from CT feed supplementation, however, only few authors studied the effects of *C. ladanifer*, as CT source, on animal production. *Cistus ladanifer* was incorporated on lamb diets, to explore its effects on productive performance, on protein digestive utilization and on meat oxidative stability and sensorial properties (Dentinho, Belo, & Bessa, 2014; Dentinho *et al.*, 2007; Francisco *et al.*, 2015; Jerónimo *et al.*, 2012; Jerónimo *et al.*, 2010). *Cistus ladanifer* incorporation on forage and concentrate-based lamb diets, did not compromised the productive performance, neither affected meat quality (Francisco *et al.*, 2015; Jerónimo *et al.*, 2010). However, when incorporated on oil supplemented diets, *C. ladanifer* protected the meat against lipid oxidation, without changes on the sensory properties of lamb meat (Francisco *et al.*, 2015; Jerónimo *et al.*, 2012). Depending to the dietary concentration, CT can reduce microbial protein synthesis and feed digestibility, and Dentinho *et al*. (2014; 2007) showed that the utilization of soybean meal supplemented with *C. ladanifer* CT extract can reduce the rumen protein effective degradability without compromising the effective digestibility in the whole digestive tract. *Cistus ladanifer* was also studied with the objective of improve the nutritional value of lamb meat lipid fraction (this point will be further described on section 1.4).

## 1. 2 Nutritional value of meat fatty acids and dietary recommendations

Meat is an essential component of human diets in several populations, providing high-quality nutrients, as protein and fat (Wyness, 2016). Consumers are becoming more aware of the relationship between diet and health, which increases the consumer attention in food nutritional value (De Smet & Vossen, 2016). Fat intake are associated to development of chronic diseases, as cardiovascular disease, cancer and diabetes type II (FAO, 2010). Global public health policies were developed to reduce the total fat, saturated fatty acids (SFA) and *trans* FA intake, being recommended the replacement of dietary SFA and *trans* FA by polyunsaturated FA

(PUFA). That process resulted in the establishment by several public organisms of dietary guidelines for intake of total fat and the main FA classes (Table 1.3).

**Table 1.3.** Recommended values for dietary intake of total fat, and fatty acids, for healthy adults

| **Total fat or fatty acid intake** | **Organism** | |
|---|---|---|
| | **EFSA[1]** | **FAO[2]** |
| Total fat | 20-35% E[a] | 20-35% E[a] |
| SFA | As low as possible | <10% E[a] |
| MUFA | No dietary reference value | up to 15-20% E[a] |
| *Trans* MUFA | As low as possible | <1% E[a] |
| PUFA | No dietary reference value | 6-11% E[a] |
| *n*-3 PUFA | No dietary reference value | 0.5-2% E[a] |
| 18:3*n*-3 | 0.5% E[a] | >0.5% E[a] |
| EPA+DHA | 250 mg/day | >250 mg/day |
| *n*-6 PUFA | No dietary reference value | 2.5-9% E[a] |
| 18:2*n*-6 | 4% E[a] | >2.5% E[a] |

[1]- European Food Safety Authorized (2010); [2]- Food and Agriculture Organization of United Nations (2010); [a]- Percentage of total energy intake

Ruminant meat is known to have higher content of SFA (45-55% of intramuscular fat), variable amounts of *trans* FA and lower PUFA contents comparing with non-ruminant meat. The consumption of ruminant edible products, as milk and meat, has been associated with detrimental effects on human health (Givens, 2010), which contribute to the negative image among consumers, that led to the reduction of ruminants meat consumption (McNeill, 2014).

The high SFA intake has been associated with increase of cardiovascular diseases, due to the increment of total cholesterol, and low density lipoprotein (LDL) cholesterol blood levels (Givens, 2009). Saturated FA effect upon serum cholesterol levels depends on individual effect of each SFA. Saturated FA between C12 and C16 has a strong cholesterol-raising effect, whereas 18:0 (stearic acid), generally the major SFA present in ruminant meat, has a neutral or slight lowering effect on LDL-cholesterol (FAO, 2010). Nutritional recommendations only indicate that SFA intake should not exceed 10% of total energy intake (FAO, 2010) or should be as low as possible (EFSA, 2010), however, individual SFA effects on cholesterol were not considered.

The PUFA and MUFA are known as beneficial for human health (Calder, 2015). Oleic acid (*c*9-18:1) is the predominant MUFA, in ruminant fat and is associated to the reduction of LDL-cholesterol (FAO, 2010). Monounsaturated FA intake recommendation can be up to 15-20% of

total energy intake, and PUFA intake between 6 and 11% of total energy intake (Table 1.3) (FAO, 2010) with *n*-6 and *n*-3 PUFA corresponding to 2.5-9% and 0.5-2% of total energy intake, respectively (FAO, 2010). The recommended minimum dietary intake of 18:2*n*-6 and 18:3*n*-3 is more than 2.5% and 0.5% of total energy intake, respectively (FAO, 2010). Linoleic (18:2*n*-6) and linolenic acids (18:3*n*-3) are essential FA for humans, as they only can be achieved by mammals though the dietary intake. The 18:2*n*-6 and 18:3*n*-3 are precursors of long chain PUFA (LC-PUFA; > C20 chain) by successive reactions of desaturation and elongation, catalyzed by Δ-5 and Δ-6 desaturases and respective elongases. The 18:2*n*-6 is the precursor of arachidonic acid (20:4*n*-6; ARA) and the 18:3*n*-3 is converted to eicosapentaenoic (20:5*n*-3; EPA), docosapentaenoic (22:5*n*-3; DPA) and docosahexaenoic (22:6*n*-3; DHA) acids. The LC-PUFA are deposited mainly in membrane phospholipids and constitute substrate for synthesis of the eicosanoids, as prostaglandins, thromboxanes or leukotrienes (Calder, 2017). Membrane phospholipids FA composition of inflammatory and immune cells determines the type of eicosanoids formed (Calder, 2017), and the eicosanoids formed from *n*-6 and *n*-3 PUFA have different biological potencies. The *n*-3 LC-PUFA have been associated with a more beneficial impact on human health compared to *n*-6 LC-PUFA, being positively linked to cognitive ability, presenting anti-inflammatory potential that can suppress chronic diseases, as rheumatoid arthritis, atherosclerosis and coronary heart disease (Calder, 2017). The *n*-6 LC-PUFA are linked to the inflammatory processes, playing an important role as precursors of inflammatory mediators. Arachidonic acid is the precursor of inflammatory eicosanoids with high pro-inflammatory potential (Calder, 2017), being associated to increased inflammatory responses in humans (Calder, 2017). The EPA and DHA have several biological actions which suggest an important role on prevention cardiovascular disease, metabolic and inflammatory diseases, cancer and neurological diseases as depression and dementia (Calder, 2017; Chikwanha, Vahmani, Muchenje, Dugan, & Mapiye, 2017). The recommended intake of EPA+DHA is more than 250 mg/day (FAO, 2010), or up to 250 mg/day (EFSA, 2010).

*Trans* FA intake is associated to deleterious effects on human health, namely the increase risk of cardiovascular diseases (Givens, 2010). *Trans* FA might come from industrial sources (industrial *trans* FA), by partial hydrogenation of vegetable oils, or from ruminant products (ruminal *trans* FA) (Aldai, de Renobales, Barron, & Kramer, 2013; Ferlay, Bernard, Meynadier, & Malpuech-Brugere, 2017). Industrial *trans* FA are rich in the *t*9-18:1 (elaidic acid) and *t*10-18:1, whereas ruminal *trans* FA are mainly composed by *t*11-18:1 (vaccenic acid), when animals fed forage diets (Scollan *et al.*, 2014). However, *t*10-18:1 is also produced by ruminants fed with grain-rich diets (Aldai *et al.*, 2013; Bessa, Portugal, Mendes, & Santos-Silva, 2005). The *trans* FA intake was recommended to be lower than 1% of total energy intake (FAO, 2010).

The consumption of ruminal *trans* FA do not contribute importantly to risks of cardiovascular disease, since they account for a small part of total FA in milk (2-5%) and meat (3-9%) (Wang, Jacome-Sosa, & Proctor, 2012). It has been described a positive association between industrial *trans* FA consumption and cardiovascular disease risk, but not with ruminal *trans* FA consumption (Aldai *et al.*, 2013; Chardigny *et al.*, 2008), moreover the *t*11-18:1 decreased atherosclerosis development, in opposition to *t*9-18:1 and *t*10-18:1 (Aldai *et al.*, 2013; Bauchart *et al.*, 2007).

The *t*11-18:1 has been suggested as beneficial *trans* FA since it is the precursor of *c*9,*t*11-18:2 (rumenic acid) in animals and man (Scollan *et al.*, 2006). Conjugated linoleic acid (CLA) are characterized by containing conjugated double bonds, that can be located at 3,5 through to 16,17; and all possible *cis*-*cis*, *cis*-*trans*, *trans*-*cis* and *trans*-*trans* combinations can be present (Jenkins *et al.*, 2008; Shingfield & Wallace, 2014). In the last years, CLA have gained interest because of their potent anti-inflammatory, immunomodulatory, anti-obese and anti-carcinogenic activity, demonstrated by cell culture studies and by laboratory animals (Kuhnt *et al.*, 2016; Shokryzadan *et al.*, 2017). Rumenic acid is the main CLA found on ruminant tissues, comprising about 80% of CLA isomers (Schmid *et al.*, 2006). The highest CLA concentrations were found in lamb meat (4.3-19.0 mg/g fat), followed by beef (1.2-10.0 mg/g fat) (Schmid *et al.*, 2006). Pork and chicken meat CLA content is usually lower than 1 mg/g fat (Schmid *et al.*, 2006). Recommendation of FAO (2010) did not, so far, establish the *c*9,*t*11-18:2 daily intake.

## 1. 3 Ruminant lipid metabolism

### 1.3.1 Ruminal lipid metabolism

Diet composition and rumen microbial metabolism are the major factors influencing FA composition of ruminant products, because the FA which reach the duodenum are, at least in part, of dietary origin as well as the result of rumen lipid metabolism. In the rumen, the dietary lipids are extensively metabolized, resulting in marked differences between FA composition of diet (mostly unsaturated FA) and FA reaching the duodenum (mostly SFA) (Jenkins *et al.*, 2008). The main types of dietary lipids entering the rumen are triacylglycerols, galactolipids and phospholipids (Jenkins *et al.*, 2008). The dietary lipids in forage consist mainly in galactolipids and phospholipids, which are rich in linolenic acid (18:3*n*-3), while cereals and plant oils contribute mainly with triacylglycerols that contain predominantly linoleic acid (18:2*n*-6) and oleic acid (*c*9-18:1) (Drackley, 2000; Harfoot & Hazlewood, 1997). The dietary lipids are transformed via two main processes, lipolysis and BH, in the rumen.

#### 1.3.1.1 Ruminal lipolysis

Dietary lipids are extensively hydrolyzed in the rumen by microbial lipases. These lipases hydrolyze the ester bonds in complex lipids, causing the release of free FA (Dawson, Hemington, & Hazlewood, 1977; Garton, Vioque, & Lough, 1961), in a process called lipolysis. Lipolysis is a pre-requisite to the next process, the BH, since the hydrogenation can only happen in free carboxyl group (Harfoot & Hazlewood, 1997). Lipolysis occurs rapidly, however, in certain conditions, as in the presence of antibiotics or low rumen pH, its extension can be decreased (Doreau & Chilliard, 1997). Lipids hydrolysis is conducted mainly by microbial lipases, although there is also the action of plant lipases (Jenkins *et al*., 2008). Among the various types of ruminal microorganisms, the bacteria are considered to be the most active in lipolysis, *Butyrivibrio fibrisolvens* and *Anaerovibrio lipolytica* are capable of hydrolyzing the ester bonds, but *B. fibrisolvens* hydrolyses phospholipids and galactolipids, and *A. lipolytic* hydrolyses triacylglicerols (Buccioni, Decandia, Minieri, Molle, & Cabiddu, 2012; Lourenco, Ramos-Morales, & Wallace, 2010).

#### 1.3.1.2 Ruminal biohydrogenation

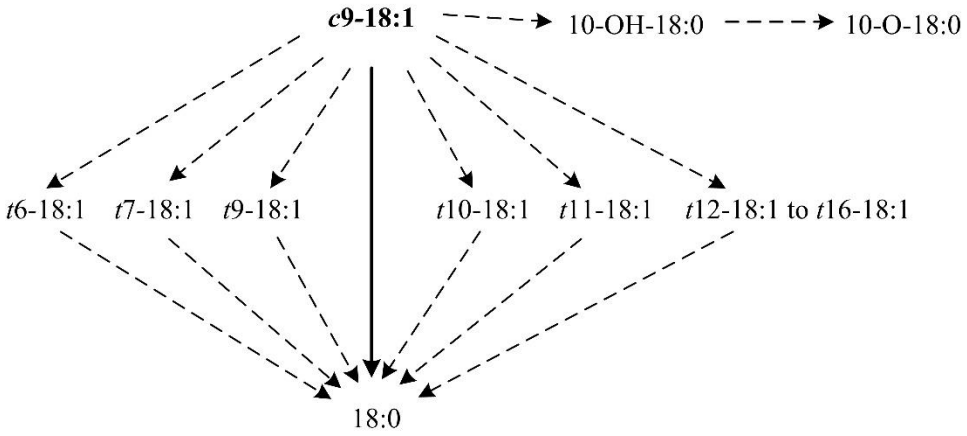
After lipolysis, the released unsaturated FA can be biohydrogenated. The ruminal BH consist in conversion of the unsaturated FA to SFA via initial isomerization to *trans* FA intermediates followed by hydrogenation of double bonds mediated by rumen microorganisms (Harfoot & Hazlewood, 1997). Two classes of microbial enzymes are involved, isomerases and redutases. The unsaturated FA are extensively biohydrogenated in rumen, and the disappearance of BH of 18:2*n*-6 and 18:3*n*-3 averages 80 and 92%, respectively (Doreau & Ferlay, 1994).

The role of BH is not entirely clear, however the most accepted theory is that ruminal BH is a detoxification strategy to prevent the toxic effects of unsaturated FA in rumen microbe (Harfoot & Hazlewood, 1997; Jenkins *et al.*, 2008; Maia *et al.*, 2010). The PUFA were found to be much more toxic than SFA (Maia *et al*., 2010), and the more unsaturated FA more toxic they are, with 18:3*n*-3 being more toxic for biohydrogenating bacteria than 18:2*n*-6 (Maia *et al.*, 2010; Maia, Wallace, Chaudhary, & Figueres, 2007). The *c*9,*t*11-18:2 is almost as toxic as 18:2*n*-6, but the *t*11-18:1 only have a little toxic effect (Maia *et al*., 2010). Ruminal microorganisms have different sensibilities to PUFA toxic effect, the *Butyrivibrio* sp. is generally more sensitive to PUFA, with *B. proteoclasticus* group being much more sensitive than *B. fibrisolvens* isolates (Paillard *et al*., 2007).

#### 1.3.1.2.1 Biohydrogenation pathways and biohydrogenation intermediates

The ruminal BH has been studied, by combining *in vitro* and *in vivo* studies, which allowed to establish some BH pathways and some BH intermediates (BI), including 18:3, 18:2 conjugated or non-conjugated and 18:1 isomers. Oleic acid (*c*9-18:1) BH (Figure 1.6**)** is generally considered as a direct conversion to 18:0 (stearic acid) (Harfoot & Hazlewood, 1997). However, recent *in vitro* studies indicated that metabolization of *c*9-18:1 can be led to the formation of numerous *trans* and *cis* 18:1 isomers, with the double bond located from C6 through C16, as well as the oxygenated stearic acid derivatives (10-OH-18:0 and 10-O-18:0, hydroxystearic and ketostearic acid, respectively), which are formed by *c*9-18:1 hydration to 10-hydroxystearic acid followed by oxidation to 10-ketostearic acid. (Jenkins, AbuGhazaleh, Freeman, & Thies, 2006; Jenkins *et al.*, 2008; Mosley *et al.*, 2006; Mosley, Powell, Riley, & Jenkins, 2002; Shingfield, Bernard, Leroux, & Chilliard, 2010). The production of *trans*-18:1 from *c*9-18:1 in the rumen is dependent on specific environmental conditions in ruminal contents that favor *cis*/*trans* isomerization.

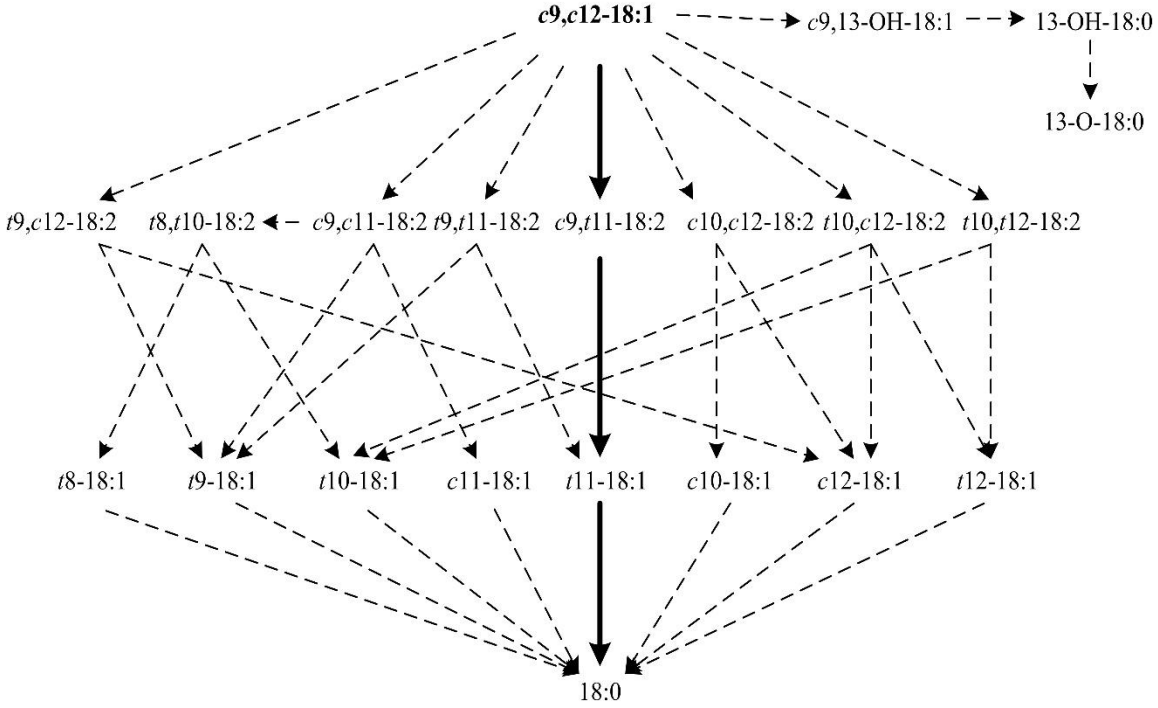
**Figure 1.6.** Biohydrogenation pathway of oleic acid (*c*9-18:1)
Arrows with solid lines highlight the main pathway and arrows with dashed lines highlight the putative pathway. Adapted from Shingfield *et al*. (2014).




The 18:2*n*-6 and 18:3*n*-3 are initially isomerized, involving the *cis*-12 double bond, with the formation of their conjugated FA, followed by hydrogenation of the double bonds (Harfoot & Hazlewood, 1997; Shingfield *et al*., 2010). The 18:2*n*-6 is isomerized to *c*9,*t*11-18:2, and other CLA isomers as well as a non-conjugated diene isomers (*t*9,*c*12-18:2) (Figure 1.7). The 18:2*n*-6 can also led to the formation of oxygenated FA (*c*9,13-OH-18:1; 13-OH-18:0 and 13-O-18:0). Then, occurs the first hydrogenation, with the production of 18:1 BI, mainly *trans*-18:1, being the *t*11-18:1 the predominant isomer, and finally, the reduction of 18:1 isomers to 18:0 (Harfoot & Hazlewood, 1997; Jenkins *et al*., 2008; Shingfield *et al*., 2010).

For all geometrical isomers possible, a total of 16 CLA isomers were identified in ruminal, omasal and duodenal digesta (Shingfield, Chilliard, Toivonen, Kairenius, & Givens, 2008; Toral, Chilliard, & Bernard, 2012). Conjugated linoleic acid formation is dependent on the composition of basal diet and FA substrates intake. The conjugated diene *c*9,*t*11-18:2, also known as rumenic acid, is the predominant CLA isomer found in rumen, milk and meat of ruminant fed diets based on forages (Jerónimo *et al.*, 2010; Toral *et al.*, 2012), and the *t*11-18:1 is the main 18:1 BI produced. However, in concentrate based diets, the BH pathways can shift and lead to the formation of *t*10,*c*12-18:2, with its conversion to *t*10-18:1 (Griinari & Bauman, 1999; Shingfield & Wallace, 2014). Piperova *et al*. (2002) reported that the relative abundance of *c*9,*t*11-18:2 decrease in high concentrate diets, inducing an increase of *t*10-18:1 flow from rumen, and demonstrated to decrease in forage:concentrate ratio from 60:40 to 25:75, promoting the ruminal shift of geometric isomers in positions 9,11 to 10,12. High levels of *t*10,*c*12-18:2 in milk (Piperova *et al*., 2002) and meat (Bessa *et al.*, 2005), has been found in ruminants fed concentrate diets.

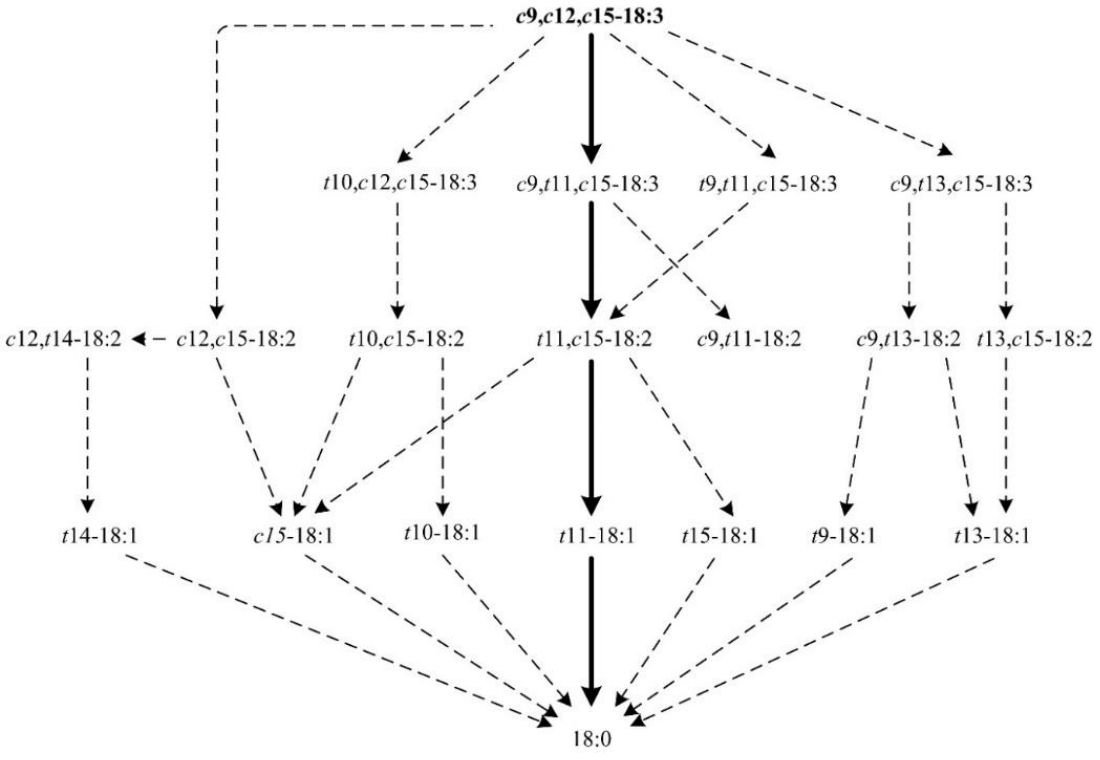
**Figure 1.7.** Biohydrogenation pathway of linoleic acid (*c*9,*c*12-18:2)
Arrows with solid lines highlight the main biohydrogenation pathway (Harfoot & Hazlewood, 1997) and arrows with dashed lines highlight the putative pathways. Adapted from Chilliard *et al*. (2007) and Shingfield *et al*. (2010).




The metabolic pathway of 18:3*n*-3 (Figure 1.8) is similar with the 18:2*n*-6. However, as 18:3*n*-3 presents three double bonds its metabolic pathways are more complex than for *c*9-18:1 or 18:2*n*-6. The first isomerization origins *c*9,*t*11,*c*15-18:3, that is sequentially reduced to *t*11,*c*15-

18:2, followed by *t*11-18:1 with 18:0 as an end product (Harfoot & Hazlewood, 1997). Further studies have demonstrated that several intermediates are formed during 18:3*n*-3 BH. Wasowska *et al*. (2006) demonstrated the formation of *c*9,*t*11,*c*15-18:3 and *t*9,*t*11,*c*15-18:3 after isomerization of 18:3*n*-3, being the *c*9,*t*11,*c*15-18:3 the main trienoic acid formed in ruminal mixed digesta. Destaillats *et al*. (2005) proposed an alternative pathway for 18:3*n*-3 which includes the initial isomerization to *c*9,*t*11,*c*15-18:3 and *c*9,*t*13,*c*15-18:3, followed by the hydrogenation to the non-conjugated dienoic isomers *t*11,*c*15-18:2 and *c*9,*t*13-18:2 and also to *t*13,*c*15-18:2. Lee & Jenkins (2011) reported the production of eight intermediate conjugated diene isomers from linolenic BH, including *c*9,*t*11-18:2. Bessa *et al*. (2007) identified the *c*12,*c*15-18:2, in meat from lambs fed lucerne supplemented with linseed oil, and proposed as a new intermediate of 18:3*n*-3 biohydrogenation, from the direct reduction of the *c*9- double bond of 18:3*n*-3. These authors suggested that *c*12,*c*15-18:2 might be hydrogenated to *c*15-18:1 or isomerized into *c*12,*t*14-18:2 and further hydrogenated to *t*14-18:1 (Bessa *et al*., 2007). According to Griinari & Bauman (1999), 18:3*n*-3 could also be converted to *t*10,*c*12,*c*15-18:3, which would be hydrogenated to *t*10,*c*15-18:2, then hydrogenated to *t*10-18:1 and finally converted to 18:0. Alves *et al*. (2014) detected and identified for the first time the *t*10,*c*15-18:2 in rumen contents, abomasal digesta and meat samples from animals fed a concentrate supplemented with 18:3*n*-3 sources.

**Figure 1.8.** Biohydrogenation pathway of linolenic acid (*c*9,*c*12,*c*15-18:3)
Arrows with solid lines highlight the main biohydrogenation pathway (Harfoot & Hazlewood, 1997) and arrows with dashed lines highlight the putative pathways. Adapted from Griinari & Bauman (1999); Bessa *et al*. (2007), Shingfield *et al*. (2010), Lee & Jenkins (2011) and Alves & Bessa (2014).



#### 1.3.1.2.2. Biohydrogenation microorganisms

The rumen microbial community comprises mainly of anaerobic bacteria, archaea, ciliate protozoa and anaerobic fungi. Bacteria play the main role in FA biohydrogenation (Jenkins *et al.*, 2008). The bacteria involved in the different steps of BH were classified into two groups based on their BH products: group A, bacteria which hydrogenate 18:2*n*-6 and 18:3*n*-3 to *t*11-18:1, and group B, bacteria that are able to saturate *t*11-18:1 to 18:0 (Harfoot & Hazlewood, 1997; Kemp, Lander, & Holman, 1984). *Butyrivibrio fibrisolvens* belongs to group A bacteria, and are the most active biohydrogenating species, however, do not form 18:0 from 18:2*n*-6 and 18:3*n*-3 (Kemp *et al*., 1984; Kepler, Hirons, McNeill, & Tove, 1966; Polan, Tove, & McNeill, 1964). The stearate producing bacteria, from group B, were initially identified as *Fusocillus* spp. (Kemp, White, & Lander, 1975), which corresponds to *Clostridium proteoclasticum* isolated by Wallace *et al*. (2006), renamed later as *B. proteoclasticus* from its 16S rRNA gene sequence (Moon *et al*., 2008), being the only known cultivated rumen bacteria identified as stearate producing bacteria. Although, the *B. fibrisolvens* and *B. proteoclasticus* are considered as play the major role on BH, recent *in vivo* microbiome studies considered that other uncultivated bacteria might be involved in BH with relevant role. Thus, bacteria phylogenetically classified as *Prevotella*, *Hungatella*, *Lachnospiraceae incertae sedis* and the unclassified *Bacteroidales*, *Clostridiales* and *Ruminococcaceae* (Buccioni *et al.*, 2017; Castro-Carrera *et al.*, 2014; Huws *et al.*, 2011) have been suspect to be involved in BH.

Up to half of the rumen microbial biomass may be protozoal in origin (Williams & Coleman, 1992). Protozoal lipids contain proportionally more unsaturated FA than the bacterial fraction (Harfoot & Hazlewood, 1997). Protozoa membranes contain a high content in *t*11-18:1 and *c*9,*t*11-18:2 (Devillard, McIntosh, Newbold, & Wallace, 2006). However, the role of protozoa on BH is not completely understood, which means the protozoa may represent an important reservoir of those FA. Protozoa are considered as having a minor role in BH, since BH in ruminal digesta was only slightly decreased after removal protozoa from the rumen (Dawson & Kemp, 1969). Anaerobic fungi form a minor part of ruminal microorganisms biomass, yet it was demonstrated that mixed ruminal fungi are capable to hydrogenate 18:2*n*-6, to *c*9,*t*11-18:2 and *t*11-18:1, but their activity is lower than *B. fibrisolvens* (Maia *et al.*, 2007; Nam & Garnsworthy, 2007).

#### 1.3.1.3 Microbial biosynthesis of the odd- and branched-chain fatty acids

The odd- and branched-chain FA (OBCFA) only occur at trace level in plants but they are synthesized at large extent by microorganisms. The OBCFA are one of the main constituents of the bacterial membrane lipids (Kaneda, 1991), and due to their low melting point, they guarantee the bacterial lipid membrane fluidity (Kaneda, 1991). Ruminant products contain OBCFA derived from ruminal bacteria activity (Vlaeminck, Fievez, Cabrita, Fonseca, & Dewhurst, 2006). The *de novo* synthesis of branched-chain FA (BCFA) in bacteria is achieved through the action of the enzyme acyl-CoA:ACP transacylase by the condensation of malonyl-coenzyme A with acyl-CoA as primer, yielding 16:0 as the dominant end product (Fulco, 1983; Kaneda, 1991). Three series of BCFA can be distinguished, even iso acids (iso 14:0, iso 16:0), odd iso acids (iso 15:0, iso 17:0) and odd anteiso acids (anteiso 15:0, anteiso 17:0). Odd-chain FA (15:0 and 17:0) are formed through elongation of propionate or valerate, whereas precursors of BCFA are branched-chain amino acids (leucine, isoleucine and valine) and their corresponding branched short-chain carboxylic acid (isobutyric, isovaleric and 2-methylbutyric acid) (Vlaeminck *et al*., 2006). The OBCFA from bacterial cell membranes have been used as marker for the rumen microbial ecosystem (Vlaeminck *et al*., 2006), since the OBCFA profile is different between bacteria species. The cellulolytic bacteria present in their lipid membrane high levels of branched-chain FA, while the amylolytic bacteria are relatively enriched in linear odd-chain FA (Vlaeminck *et al*., 2006).

### 1.3.2 Synthesis of fatty acids in muscle

Meat lipids consists of phospholipids which are components of the cell membranes, and triacylglycerols mostly deposited in the adipocytes (Raes, De Smet, & Demeyer, 2004). The muscle phospholipids content is almost constant and independent of the total fat content, while the intramuscular triacylglycerols content is less constant and strongly related to the total fat content (Bessa, Alves, & Santos-Silva, 2015; Raes *et al.*, 2004; Wood *et al.*, 2008).

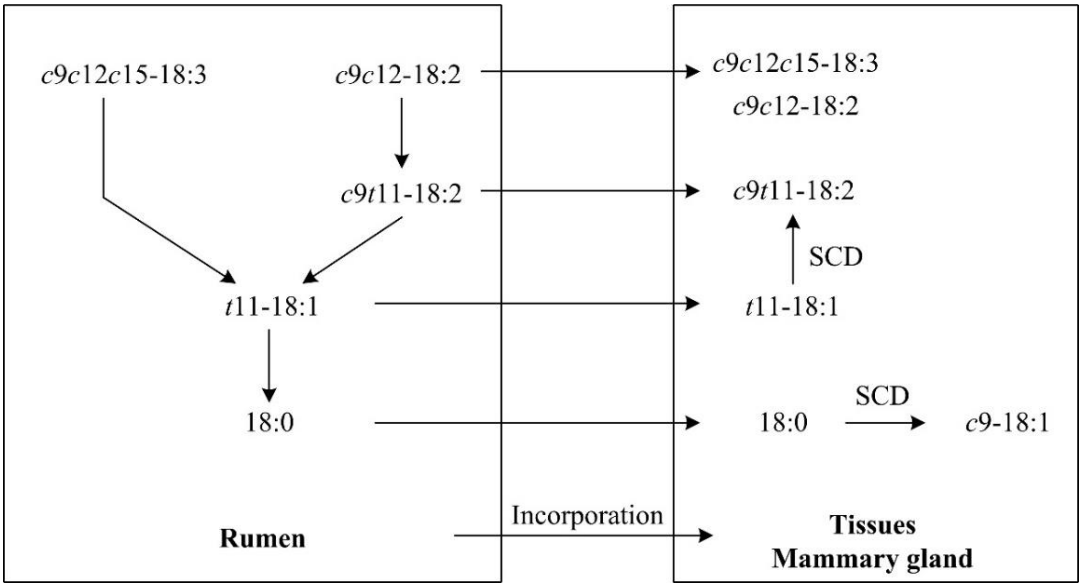
Fatty acids incorporated in muscle lipids may be derive from preformed circulating FA or by *de novo* synthesis. The biohydrogenation products (BI and 18:0) can be incorporated into triacylglycerols or phospholipids or can be elongated and/or desaturated before deposition (Drackley, 2000). *De novo* FA synthesis occurs in the cytosol and is a sequential cyclical process in which acetyl (2-carbon) units are added to a primer molecule, frequently acetyl-CoA (Drackley, 2000). *De novo* FA synthesis yields 16:0 as the main end product that can be serve as a substrate for elongation and/or desaturation.

Stearoyl-CoA desaturase (SCD) enzyme also known Δ9-desaturase plays a key role in the lipid metabolism, since add a *cis* double bond between carbons 9 and 10 of the FA chain. The substrates of this reaction are mostly 16:0 and 18:0, being converted into *c*9-16:1 (palmitoleic acid) and *c*9-18:1, respectively (Ntambi & Miyazaki, 2004). However, other saturated FA and unsaturated FA can serve as substrates, including *t*11-18:1. Vaccenic acid, formed during ruminal BH of 18:2*n*-6 and 18:3*n*-3, is the precursor of *c*9,*t*11-18:2, and in tissues is converted by SCD in *c*9,*t*11-18:2 (Figure 1.9). The endogenous synthesis via SCD represents the predominant source of CLA in ruminant tissues (Bauman, Baumgard, Corl, & Griinari, 1999). According to Palmquist *et al*. (2004), about 80% of the *c*9,*t*11-18:2 deposited in the lamb tissue is originated by endogenous desaturation of *t*11-18:1 produced in rumen.

The factors that might affect the activity and expression of SCD, as dietary, hormonal, genetic and environmental factors, might also affect the content of *c*9,*t*11-18:2 and the *t*11-18:1 availability. Although these factors were studied mainly in laboratory animals and humans (Ntambi & Miyazaki, 2004), being the information about regulation of expression and activity of SCD in ruminants very scarce.

**Figure 1.9.** Pathways of *c*9,*t*11-18:2 biosynthesis

Adapted from Palmquist *et al.* (2004)




## 1. 4 Dietary strategies to modulate the ruminal biohydrogenation to improve the fatty acid composition of ruminant products

Ruminant edible products are characterized by being rich in SFA, due to the action of ruminal microorganisms on the dietary lipids that are converted in high amounts of SFA (Jenkins *et al.*,

2008). In ruminant, the BH of unsaturated FA constitute a limitation of PUFA enrichment in ruminant products. Thus, modifying the ruminal BH of dietary PUFA through animal diets may be a way to improve the healthiness of ruminant fat, enhancing the yield in benefic FA, such as *t*11-18:1, *c*9,*t*11-18:2 and PUFA, in ruminant meat. Several dietary strategies can be used to improve the nutritional value of ruminant products, which may involve the ruminal BH manipulation of dietary PUFA and could promote the absorption and deposition of the target FA in intramuscular fat (Bessa *et al.*, 2015; Palmquist, Lock, Shingfield, & Bauman, 2005). Thus, in addition to dietary strategies aimed to increase the PUFA content, which intend to inhibit the lipolysis and the initial ruminal BH steps, arise new strategies that aim to increase the *c*9,*t*11-18:2 and *t*11-18:1 content on ruminant fat, which intend to inhibit the last reductive step of BH, without limiting the conversion of dietary unsaturated FA to *c*9,*t*11-18:2 and to *t*11-18:1 (Bessa *et al.*, 2015; Lourenco *et al.*, 2010)

On the next sub-sections are presented several factors that can modulate the ruminal BH, such as the amount and type of lipid supplement, as basal diet and plant secondary metabolites.

### 1.4.1 Lipid supplementation

The inclusion of lipid sources in ruminant diets can be used to manipulate ruminal BH due to their antimicrobial effect. The antimicrobial effect of dietary lipids is associated with the degree of unsaturation of the FA, and it will change the microbial ecology and consequently the ruminal BH (Maia *et al.*, 2010; Maia *et al.*, 2007). Vegetable oils rich in PUFA, when included in diets, increases the rumen outflow of dietary PUFA, individual BI (as *t*11-18:1 and *c*9,*t*11-18:2) and the end product (18:0) (Loor, Ueda, Ferlay, Chilliard, & Doreau, 2004; Shingfield *et al*., 2008). Oilseed products (linseed, soybean and sunflower oils and seeds) increased *trans*-18:1 FA. However, it must be noted that the effect on *t*11-18:1 is highly dependent of the forage:concentrate ratio of the diet (see below on 1.4.2 section). Oils rich in linoleic acid (sunflower and soybean) have been more effective in enhancing milk *t*11-18:1 and *c*9,*t*11-18:2 than oils rich in linolenic acid (linseed) (Bu, Wang, Dhiman, & Liu, 2007).

Lipid sources rich in 18:2*n*-6 and 18:3*n*-3, when used as diet supplement can improve the *c*9,*t*11-18:2 content of ruminant products (Schmid *et al.*, 2006). The supplementation of sunflower and linseed oil in a lucerne basal diet increased *c*9,*t*11-18:2 content in muscle, being higher with sunflower oil than with linseed oil supplementation (Bessa *et al*., 2007), this could be explained by the fact that during ruminal BH of 18:3*n*-3 is not produced *c*9,*t*11-18:2, being therefore less effective in the increase of *c*9,*t*11-18:2 in fat. The dietary replacement of

sunflower oil with linseed oil decreased the *c*9,*t*11-18:2 content in lamb meat, being the maximum of *c*9,*t*11-18:2 content achieved with 100% of sunflower oil, decreasing linearly by linseed replacement (Jerónimo, Alves, Prates, Santos-Silva, & Bessa, 2009). These authors also demonstrated that the utilization of blend oils (sunflower and linseed oils) is an effective approach for the simultaneous enrichment of *c*9,*t*11-18:2 and *n*3-PUFA in lamb meat, achieving the maximum of both FA at 52% of replacement of sunflower with linseed oil (Jerónimo *et al.*, 2009).

### 1.4.2 Basal diet

Basal diet has a large effect on ruminal BH and consequently on ruminant products FA composition. Forages and pasture are considered one of the best strategies to increase the content of *c*9,*t*11-18:2, *t*11-18:1 and *n*-3 PUFA in milk and meat, because forages are a major source of 18:3*n*-3 (Scollan *et al.*, 2014). However, feeding growing ruminants with increasing proportion of concentrate in diet change the ruminal BH pathways favoring the production of *t*10,*c*12-18:2 and *t*10-18:1 (designed as *t*10-shift) instead *c*9,*t*11-18:2 and *t*11-18:1 (Griinari & Bauman, 1999). In forage or pasture fed ruminants, the *t*11-18:1 represents about 55-60% of total *trans* 18:1, could this value be higher (up to 70%) when the diets were supplemented with vegetable oils (Alfaia *et al.*, 2009; Bessa *et al.*, 2007; Bessa *et al.*, 2005). Type of basal diet when supplemented with vegetable oils led to a different FA composition. Indeed, the supplementation with 10% of soybean oil amplified the differences between lucerne and concentrate (Bessa *et al.*, 2005). The oil-supplemented lucerne increased the content of *c*9,*t*11-18:2 and *t*11-18:1. However, the supplementation of concentrate with soybean oil resulted in an increase of *t*10,*c*12-18:2 and *t*10-18:1 in lamb muscle (Bessa *et al.*, 2005).

Botanical composition of the forages also affects FA metabolism in rumen and forages may provide added benefits (Lourenco *et al.*, 2010). Some studies associated the presence of plant secondary compounds in botanically diverse pastures with the effects on microbial BH activity in rumen, promoting the accumulation of *c*9,*t*11-18:2 and *t*11-18:1 on the ruminant products (Lourenco, Van Ranst, Vlaeminck, De Smet, & Fievez, 2008; Priolo *et al.*, 2005).

### 1.4.3 Plant secondary compounds

There is an increasing interest over the plant secondary compounds present in plants, as shrubs, bushes, pastures and in agro-industrial by-products, as tools for modulate ruminal BH and

improve ruminant products quality. Plant secondary compounds, such as phenolic compounds, essential oils and condensed tannins have been studied, both *in vitro* and *in vivo*, to evaluate the effects by which plant secondary compounds modulate the ruminal BH and consequently promote products quality.

According to the actual knowledge about the *trans*-18:1 and *c*9,*t*11-18:2 FA effects on human health, is desirable to improve the amount of *c*9,*t*11-18:2 and *t*11-18:1 on the ruminant products and decrease the amount of *t*10-18:1 (Aldai *et al.*, 2013). Several studies demonstrate that plant secondary compounds have the ability to influence the ruminal microbes and have potential to modulate the ruminal BH, so the effect of plant secondary metabolites on FA profile, both *in vitro* and *in vivo* and on ruminal microorganisms, will be described in the next sections (1.4.3.1, 1.4.3.2 and 1.4.3.3).

### 1.4.3.1 Influence of plant secondary compounds on ruminal biohydrogenation – *in vitro* studies

The BH process is largely influenced by forage species and the composition of the plants (Lourenco *et al*., 2008). The effect of twenty-seven tropical plants, with different phenolic profiles, on ruminal BH were studied, and the authors observed that moderate phenolic content (73 g gallic acid equivalent/kg DM) of *Persea americana* leaves (avocado) changed the BH pattern, increasing the bypass of PUFA and the *t*11-18:1 production and decreasing the 18:0 content (Jayanegara, Kreuzer, Wina, & Leiber, 2011). In this study, a significant negative correlation between phenolic content and the disappearance of 18:3*n*-3, 18:2*n*-6 and 18:1*n*-9 was found, suggesting that phenols may reduce the ruminal BH from the first step (Jayanegara *et al.*, 2011).

Recent studies showed that *Carica papaya* leaf or its extracts, rich in phenolic compounds and CT, decreased the BH of C18 PUFA, without changing the *c*9,*t*11-18:2, *t*11-18:1 and 18:0 production (Jafari, Goh, Rajion, Jahromi, & Ebrahimi, 2016a; Jafari, Meng, Rajion, Jahromi, & Ebrahimi, 2016b). However, when papaya leaf methanolic extract was incubated with three different doses (20, 40 and 60 g/kg DM), decreased the BH of C18 PUFA compared to control, and resulted in a higher concentration of *t*11-18:1 (increase of 41% for 60 g/kg DM dose) and *c*9,*t*11-18:2 (increase of 23%), without significant effect on 18:0 production (Jafari *et al*., 2017). Miri *et al*. (2013) showed that plant methanolic extracts, from *Azaditachita indica*, *Allium sativum* and *Cuminum cyminum*, at the level of 1 mg/mL improved *c*9,*t*11-18:2 production (59, 58 and 65%, respectively) and the extract of *Terminalia chebula* at level of 2 mg/mL increased

the *t*11-18:1 production by 29% in ruminal fluid when compared with control. According to Durmic *et al*. (2008), from 91 plant ethanolic extracts and essential oil, only a few plants, as *Acacia iteaphylla*, inhibited the saturation of 18:2*n*-6 and the 18:0 formation, increasing the accumulation of *t*11-18:1.

The effect of essential oil and compounds, extracted from essential oil, on ruminal BH has also been studied. Lourenço *et al*. (2008) showed that eugenol (250 mg/L) caused minor inhibition of BH process, when incubated *in vitro*, whereas, cinnamaldehyde (500 mg/L) induced an overall inhibition of BH, and the microbial biomass activity, resulting in a great accumulation of BI, other than *c*9,*t*11-18:2 and *t*11-18:1. Siurana *et al*. (2018) showed consistent results with an increase of 18:3*n*-3 and an decrease of 18:0, without significant accumulation of *t*11-18:1 and *c*9,*t*11-18:2. These differences in the results could be due to the different tested concentrations (250 *vs*. 500 mg/L), or to the molecule type (eugenol *vs*. cinnamaldehyde). Gunal *et al*. (2013; 2014) tested the effect of different essential oils on *in vitro* ruminal BH, with three doses (125, 250 and 500 mg/L), and showed that the concentration of 18:0 and *trans*-18:1 decreased with the essential oils inclusion regardless of the dose level, and the *c*9,*t*11-18:2 concentration increased with cedar wood and cinnamon oil, at 500 and 250 mg/L dose, respectively. Some essential oils increased the 18:2*n*-6 concentration when added at 500 mg/L. These results suggest the shift in the BH pathways with formation of unidentified BI. However, Mandal *et al*. (2016) observed that the inclusion of buds and seeds extracts from three plants (buds and seeds) rich in essential oil (*Syzygium aromaticum* buds, *Allium sativum* buds and *Coriandum sativum* seed) did not affected the *c*9,*t*11-18:2 and *t*11-18:1 concentration at the tested levels (1 and 2% of extract). The effects of essential oil are variable, depending on the composition, the tested dose, the basal diet and the possible adaptation of ruminal microbes to essential oil presence (Benchaar, McAllister, & Chouinard, 2008).

Condensed tannins incubation with ruminal fluid has been described as effective on the inhibition of the last step of BH (Khiaosa-Ard *et al.*, 2009; Mandal *et al.*, 2016; Vasta, Makkar, Mele, & Priolo, 2009a). Condensed tannins extract from carob (*Ceratonia silique*), *Acacia cyanophylla*, quebracho (*Schinopsis lorentzii*) (Vasta *et al.*, 2009a), *Acacia mearnsii* (Khiaosa-Ard *et al.*, 2009) and *Artocarpus heterophyllus* (Mandal *et al*., 2016) inhibited BH last step, with accumulation of *t*11-18:1 (respectively, more 29, 61 and 7%, when compared with respective control), without affecting the *c*9,*t*11-18:2 production. The effects of two different tannins sources: condensed (*Schinopsis lorentzii* - quebracho) and hydrolysable (*Castanea sativa* - chestnut) extracts, on *in vitro* BH were explored on rumen bacteria, during 18h of incubation (Buccioni, Minieri, Rapaccini, Antongiovanni, & Mele, 2011). Solid-associated

bacteria (SAB) contained higher amounts of *t*11-18:1 and *c*9,*t*11-18:2 than liquid-associated bacteria (LAB), that presented higher amounts of 18:0. The increase of *t*11-18:1 and *c*9,*t*11-18:2, in SAB, were more accentuated with the lower dose tested ( more 28 *vs*. 27%, respectively for 49 *vs*. 82 g/kg DM), except for *c*9,*t*11-18:2 that increased with 82 g/kg DM of quebracho (71% more than control), for 18h of incubation. These effects were found for the higher incubation times (12 and 18h) and suggest an inhibition of the last step of BH (Buccioni *et al.*, 2011). However, Carreño *et al*. (2015) demonstrated a general BH inhibition, instead of the negative effect on the conversion of *t*11-18:1 to 18:0, when studied different doses (20 to 80 g/kg DM) of CT extracts (*Schinopsis lorentzii* – quebracho and *Vitis vinifera* – grape) and hydrolysable tannins extracts (*Castanea sativa* – chestnut and *Quercus robe* plus *Q. petraea* – oak). Both tannins type promoted an increase of 18:2*n*-6 and 18:3*n*-3 levels in ruminal fluid (increase of 37 and 39%, respectively) with low and moderate doses. The *c*9,*t*11-18:2 concentration increased with quebracho (more 38%) and chestnut (more 52%) at 60 g/kg DM, while 80 g/kg DM was needed to increase *c*9,*t*11-18:2 concentration with grape (more 56%) and oak extracts (more 41%). All extracts and doses (except quebracho at 80 g/kg DM) decrease the 18:0 concentration, while the *t*11-18:1 concentration tended to increase with 60 and 80 g/kg DM of grape and chestnut and 20 g/kg DM of oak extracts. Similarly, Minieri *et al*. (2014) analyzed SAB fraction and demonstrated that quebracho tannins extract inhibited the first steps of the BH, with reduction of the 18:2*n*-6 and 18:3*n*-3 BH and 18:0 production, but without the accumulation of *t*11-18:1. However, the presence of CT increased the proportion of *c*9,*t*11-18:2 when incubated with soybean oil, which is rich in 18:2*n*-6. In the last year, a study with tannins extracts (quebracho, chestnut, grape seed and *C. ladanifer*) demonstrated the same effect, the stimulation of the first step of BH, without inhibition of the 18:0 production (Costa *et al.*, 2017a). This effect was observed with grape seed and *C. ladanifer* extracts, which presented a higher *c*9,*t*11-18:2 and *t*11-18:1 production and more 18:2*n*-6 and 18:3*n*-3 BH (1.4- and 1.3-fold, respectively) than the other extracts. The inconsistency of the results obtained with tannins can be due to CT composition, CT concentration, substrate/feed used, as well as the applied methodologies (*in vitro* incubations). In fact, CT comprise a very wide and heterogeneous group of phenolic compounds with different chemical and structural features (Mueller-Harvey, 2006) and molecular weights. This structural diversity would account for major variations in their ability to bind to other molecules or to affect microorganisms, and consequently its effects upon ruminal BH.

### 1.4.3.2 Influence of plant secondary compounds on ruminal biohydrogenation – *in vivo* studies

Biohydrogenation modulation by plant secondary compounds affects the FA profile of the rumen, abomasal digesta, meat and milk. The supplementation of Barbarine lamb' diet with *Artemisia herba alba* essential oils (400 ppm) led to an increase of *t*11-18:1, *c*9,*t*11-18:2, 18:3*n*-3 and total MUFA concentrations in *longissimus muscle* (respectively, more 48, 36, 43 and 23% compared to the control), without effect on the 18:0 content (Vasta *et al.*, 2013). In the same work, *Rosmarinus officinalis* essential oil did not affect ruminal BH (Vasta *et al.*, 2013). These results suggest that the supplementation with *Artemisia* essential oil could induced a BH impairment with the consequent accumulation of the BI. The lack of effects of rosemary oil could be due to the differences in chemical composition of the two essential oils, as the antimicrobial activity of essential oil depends on the chemical structure of terpenes (the main constitutes of essential oils), being some terpenes eventually degraded by ruminal microorganisms. Rosemary oil compounds might be less efficient than the artemisa oil compounds in impairing BH, or could be degraded in rumen (Vasta *et al.*, 2013). Mandal *et al*. (2014) demonstrated that the feeding with *Syzygium aromaticum* essential oil (2.5 g/kg DM) might improve the beneficial FA concentrations in meat without any adverse effect on digestibility and growth performance in Black Bengal goats. In this study, the essential oil decreased the total SFA and 18:0 proportion (decrease of 21 and 14%, respectively) and increased the PUFA proportion (more 22%), and tended to increase the *t*11-18:1 and *c*9,*t*11-18:2 concentrations in both muscle and adipose tissues.

In what concerns to CT, when lambs fed sulla (*Hedysarum coronarium*; 1.8% of DM of CT) were compared to lambs fed sulla with polyethylene glycol (PEG, a tannins binding agent), Priolo *et al*. (2005) were not able to evidence any specific CT effect upon muscle FA composition. Quebracho tannins (*Schinopsis lorentzii*) were studied *in vivo*, and when incorporated into a concentrate-based diet, quebracho tannins resulted in an accumulation of *t*11-18:1, *c*9,*t*11-18:2 and total PUFA, and in reduction of 18:0 in ruminal fluid and lamb meat, suggesting that quebracho tannins reduce the ruminal BH, principally its last step (Vasta *et al.*, 2010a; Vasta *et al.*, 2009b; Vasta *et al.*, 2009c). Contrarily, when incorporated into a herbage-based diets, quebracho tannins were ineffective to modulate the BH (Vasta *et al.*, 2010a; Vasta *et al.*, 2009b; Vasta *et al.*, 2009c). Similar results were obtained with incorporation of cumin seed extract (1.27% DM) in concentrate-based diets, which increased *c*9,*t*11-18:2 and *t*11-18:1 in goats rumen, when compared to cumin-free diet (Miri, Ebrahimi, & Tyagi, 2015). The cumin also increased the 18:2*n*-6 and 18:3*n*-3 concentration and decreased the 18:0 ruminal content,

which reflected a possible inhibition or retardation in BH pathways (Miri *et al.*, 2015). Vasta *et al*. (2007) fed lambs with concentrate supplemented with carob (*Ceratonia siliqua*, 45% DM), and carob plus PEG, reported that the intramuscular fat of lambs fed carob diet presented lower concentration of *c*9,*t*11-18:2 and *t*11-18:1 compared with meat from lamb fed carob plus PEG or lamb fed concentrate-based diet. On the contrary, Gravador *et al*. (2015) showed that carob pulp incorporation, up to 35% in concentrate-based diets could reduce BH of dietary PUFA with higher deposition of 18:2*n*-6 (28%), 18:*3n*-3 (61%) and *c*9,*t*11-18:2 (49%) content in the muscle, and lower 18:0 but similar *t*11-18:1 concentration than the carob-free diet. This results inconsistency clearly suggests an unclear effect of carob on BH.

*Cistus ladanifer* (leaves and soft stems), used as CT source (250 g/kg DM, 2.5% of CT), when incorporated in dehydrated lucerne lamb diet, combined with 6% of sunflower and linseed oil (1:2, v/v), enhanced the *c*9,*t*11-18:2 (1.4-fold) and *t*11-18:1 (1.8-fold) content in lamb meat, and decreased the 18:0 (1.4-fold) content in abomasal digesta (Jerónimo *et al.*, 2012; Jerónimo *et al.*, 2010), suggesting the inhibition of the last step of BH. In the same studies, the grape seed extract (25 g/kg DM, 2.4% of CT) inclusion had a minor effect on FA composition of abomasal digesta and muscle. The higher *t*11-18:1 availability in tissues for endogenous synthesis led to a higher *c*9,*t*11-18:2 deposition in muscle of lambs fed oil supplemented *C. ladanifer* diets (Jerónimo *et al.*, 2012; Jerónimo *et al.*, 2010). These distinct effects between the two CT sources could be due to differences on CT composition, structure and/or the presence of other plant secondary metabolites present in *C. ladanifer* leaves and soft stems. More recently, Francisco *et al*. (2016) showed that inclusion of *C. ladanifer* (20% DM) and oil (4% of soybean and linseed, 1:2, v/v) in 1:1 forage:concentrate proportion diets, increased the accumulation of *t*10-18:1 in muscle instead of *t*11-18:2 and *c*9,*t*11-18:2 accumulation. These negative effect on lamb meat profile can be due to exacerbation of ruminal *t*10-shift, and are probably due to lower forage:concentrate proportion diet (1:1), than the ones used (9:1) in previous work by Jerónimo *et al*. (2010). The same effect, occurrence of *t*10-shift with accumulation of *t*10-18:1 at the expense of *t*11-18:1, was observed by Alves *et al*. (2017) in rumen, abomasum and plasma of the same lambs. As the *t*10-18:1 might have detrimental health effects on consumers, it should be prudent considered the supplementation of high *C. ladanifer* CT and PUFA oil doses in high concentrate diets of lambs.

Beyond the rumen, abomasal digesta and meat, milk FA composition is also affected by CT. Milk from ewes grazing sulla (*Hedysarum coronarium*; approx. 2.5% CT) is low in *c*9,*t*11-18:2 and *t*11-18:1 (less 1.7-fold, for both FA), but presented high beneficial FA (18:2*n*-6 and 18:3*n*-3), when compared to milk from ewe fed sulla with PEG, which led to higher *c*9,*t*11-18:2 and

*t*11-18:1 concentrations (Cabiddu *et al*., 2009). On the contrary, Buccioni *et al*. (2015) demonstrated that quebracho and chestnut tannins extracts in soybean oil supplemented diets induced significant alterations on milk FA composition. The quebracho diet was more effective in changing the ruminal BH, led to *c*9,*t*11-18:2 and *t*11-18:1 accumulation in milk than in control or chestnut diets. Supplementation of dairy goat diets with agro-industrial by-products (pomegranate seed pulp, pistachio hulls and tomato pomace) enhanced the *c*9,*t*11-18:2 and *t*11-18:1 contents in milk, improving the quality of milk fat properties (Razzaghi *et al*., 2015). The inconsistent results observed in milk FA composition can be due to the same factors that were indicated in the last sections (for both *in vitro* and *in vivo* studies).

#### 1.4.3.3 Influence of plant secondary compounds on ruminal microorganisms

Essential oils are used in ruminant diets as modifiers of rumen fermentation due to its antimicrobial properties. Essential oils adsorb on bacteria cell membranes causing conformational changes, being also able to bind enzymes, impeding bacterial growth and activity (Calsamiglia, Busquet, Cardozo, Castillejos, & Ferret, 2007). Incubation of a commercial blend of essential oils (blend of natural compounds that includes thymol, eugenol, vanillin and limonene, at concentrations of less than 100 ppm), in pure cultures, inhibited the growth of some ruminal bacteria, being the *Prevotella ruminicola*, *Clostridium sticklandii* and *Peptostreptococcus anaerobius* the most sensitive (McIntosh *et al.*, 2003). While, *Streptococcus bovis* was the most resistant bacteria, and *B. fibrisolvens* and *Ruminococcus flavefaciens* presented an intermediate sensibility to essential oils (McIntosh *et al.*, 2003). However, some species as *P. ruminicola* and *P. bryantii* became adapted and were able to grow in the presence of higher concentrations of essential oils, while *C. sticklandii* and *P. anaerobius* remained sensitive (McIntosh *et al*., 2003). The abundance of *R. flavefaciens* increased in the presence of peppermint oil (0.33 mL/L), but the population density decreased with the higher oil levels (>1 mL/L) (Agarwal, Shekhar, Kumar, Chaudhary, & Kamra, 2009). The growth of *B. fibrisolvens* and *B. proteoclasticus* was inhibited by essential oil extracted from *Agonis fragrans*, *Eucalyptus plenissima*, *Eucalyptus staigeriana*, *Lavandula intermedia*, *Leptospermum petersonii*, *Malaleuca capreolata*, *Malaleuca ericifolia* and *Santalum spicatum* (Durmic *et al*., 2008). While the supplementation of dairy cows diets with 1g/day of cinnamaldehyde had no effect on total protozoa, but increased the number of *Isotricha* (Benchaar *et al.*, 2008).

Tannins are generally considered as inhibitory to the growth and activities of rumen microorganisms, which might be also responsible for BH modulation. The effect of tannin complexation with proteins and bacterial cell membranes results in both extracellular enzyme inhibition and unavailability of substrates for digestion, which will lead to inactivation of microorganisms and ultimately to cell death (Smith, Zoetendal, & Mackie, 2005). Sainfoin (*Onobrychris viciifolia*) CT reduced the growth of proteolytic bacteria (*B. fibrisolvens*, *R. amyliphilus* and *S. bovis*), and a strain of *P. ruminicola* was tolerant (Jones, McAllister, Muir, & Cheng, 1994). McSweeney *et al*. (2001) demonstrated that inclusion of 30% of *Calliandra* leaves reduced total cellulolytic bacteria (*Fibrobacter succinogenes* and *Ruminococcus* spp.) without effect upon total proteolytic bacteria. These controversy results indicate that different tannins sources have different actions on microbial species.

Tannins effects in ruminal microorganisms can be influenced by the tannins structure and tannins molecular weight. The *Dorycnium rectum* CT fractions with different molecular weights (low, medium and high molecular weight), inhibited *B. proteoclasticus* growth at concentrations range 100 to 300 mg/L of *in vitro* medium. Although, low and medium molecular weights fractions inhibited *B. fibrisolvens* growth, at the same concentrations, *B. fibrisolvens* and *B. proteoclasticus* growth was stimulated with the concentration of 100 mg/L of the high molecular weight fraction (Sivakumaran *et al*., 2004). In a recent study, Saminathan *et al*. (2016) reported that the bovine rumen microbiota (control group) were dominated by the genus *Prevotella* (36% of relative abundance), unclassified *Clostridiales* (6%), *Treponema* (4.5%), *Ruminococcus* (4%), *Fibrobacter* (2.5%), *Butyrivibrio* (2.4%), *Anaeroplasma* (2.2%), *Proteiniclasticum* (2%), *Succiniclasticum* (2%), *Paraprevotella* (1.5%), *Ruminobacter* (1.2%) and *Streptococcus* (1%), and other genera in minor abundance (<1% of relative abundance). The incorporation of *Leucaena leucocephala* CT fractions with different molecular weights (F0, the unfractionated fraction and F1-F5, where F1 had the higher molecular weight and the F5 the lower one) demonstrated that the predominant *Prevotella* and unclassified *Clostridiales* genus decreased with increasing molecular weight fractions. Higher molecular weight fractions also decreased *Ruminococcus*, (F1-F3) and *Streptococcus* (F1 and F2) and the *Butyrivibrio* was inhibited by F0, F2 and F5 fractions. In contrast, the relative abundance of *Fibrobacter* increased with the five CT fractions (F1-F5) and *Acinetobacter* and *Selenomonas* increased with the higher molecular weight fractions (F1 and F2). This result confirmed that CT type, molecular weight and chemical structure can influence the growth of rumen cellulolytic bacteria. The inclusion of high molecular weight CT fractions enhanced the *Fibrobacter* relative abundance, which could maintain the normal fibrolytic activity, while led to a decrease of the *Ruminococcus*, the dominant cellulolytic bacteria. The *Fibrobacter*, *Acinetobacter* and

*Selenomonas* growth with higher molecular weight CT suggest that these bacteria are tannin-resistant. Other tannin-resistant bacteria were also isolated from cattle rumen, as *Proteobacteria*, *Streptococcus* and *Butyrivibrio* spp. (Belenguer *et al*., 2010; Jones *et al*., 1994). The enhancement in tannin-resistant bacteria could be due to the action of higher molecular weight fractions, as phytofactors which limit other microorganisms growth, causing an increase in substrate availability for these bacteria, or by the ability of the tannin-resistant bacteria to degrade tannins fractions, and use them as an energy source (Saminathan *et al*., 2016). These authors also demonstrated that the rumen bacterial composition differed between treatments, and ruminal bacteria communities from F1 fraction were distinctly separated from those the control and from the lower molecular weight fractions by principal component analysis. This is corroborated by Buccioni *et al*. (2017), who showed that ruminal communities from tannin treatments (quebracho and chestnut) were separated from the control when canonical correspondence analysis were carried out to find connection between ruminal communities and ruminal FA profile. The total bacteria, and *Butyrivibrio* group communities from tannin diets were positively correlated to 18:2*n*-6, 18:3*n*-3, *c*9,*t*11-18:2 and *t*11-18:1 production, whereas the total bacterial, and *Butyrivibrio* group communities from control were positively correlated with 18:0 production. The two types of tannins increased the 18:2*n*-6, 18:3*n*-3, *c*9,*t*11-18:2 and *t*11-18:1, being more evident in presence of quebracho and this was associated with higher intensity of seven DNA bands in the *Butyrivibrio* group that belong to genera *Hungatella*, *Ruminococcus*, *Eubacterium* and unclassified *Lachnospiraceae*. This confirms the association of *Butyrivibrio* group with the BH pathways. However, as some DNA bands were related with unclassified *Lachnospiraceae* species, this the presence of unknown species, which might play a role in BH of dietary PUFA (Buccioni *et al.*, 2017).

Vasta *et al*. (2010b) showed that quebracho tannins increased the *B. fibrisolvens*, and decreased *B. proteoclastiscus* relative abundance in rumen *in vivo* with effect on BH FA, which suggested that conversion of *t*11-18:1 to 18:0 was inhibited by tannins, and most probably due to the decrease of *B. proteoclasticus* relative abundance with quebracho tannins incorporation, since *B. proteoclasticus* is considered as stearate-producer. The authors also reported the increase on total protozoa number in lambs supplemented with tannins. The effects of tannins on rumen protozoa are controversy, some studies reported that tannins have no effects on rumen protozoa, others showed a reduction of protozoal number, and others demonstrated an increase of total protozoa population (Benchaar *et al.*, 2008; Jafari *et al.*, 2016a; Jafari *et al.*, 2016b; Szczechowiak *et al.*, 2016; Vasta *et al.*, 2010b).

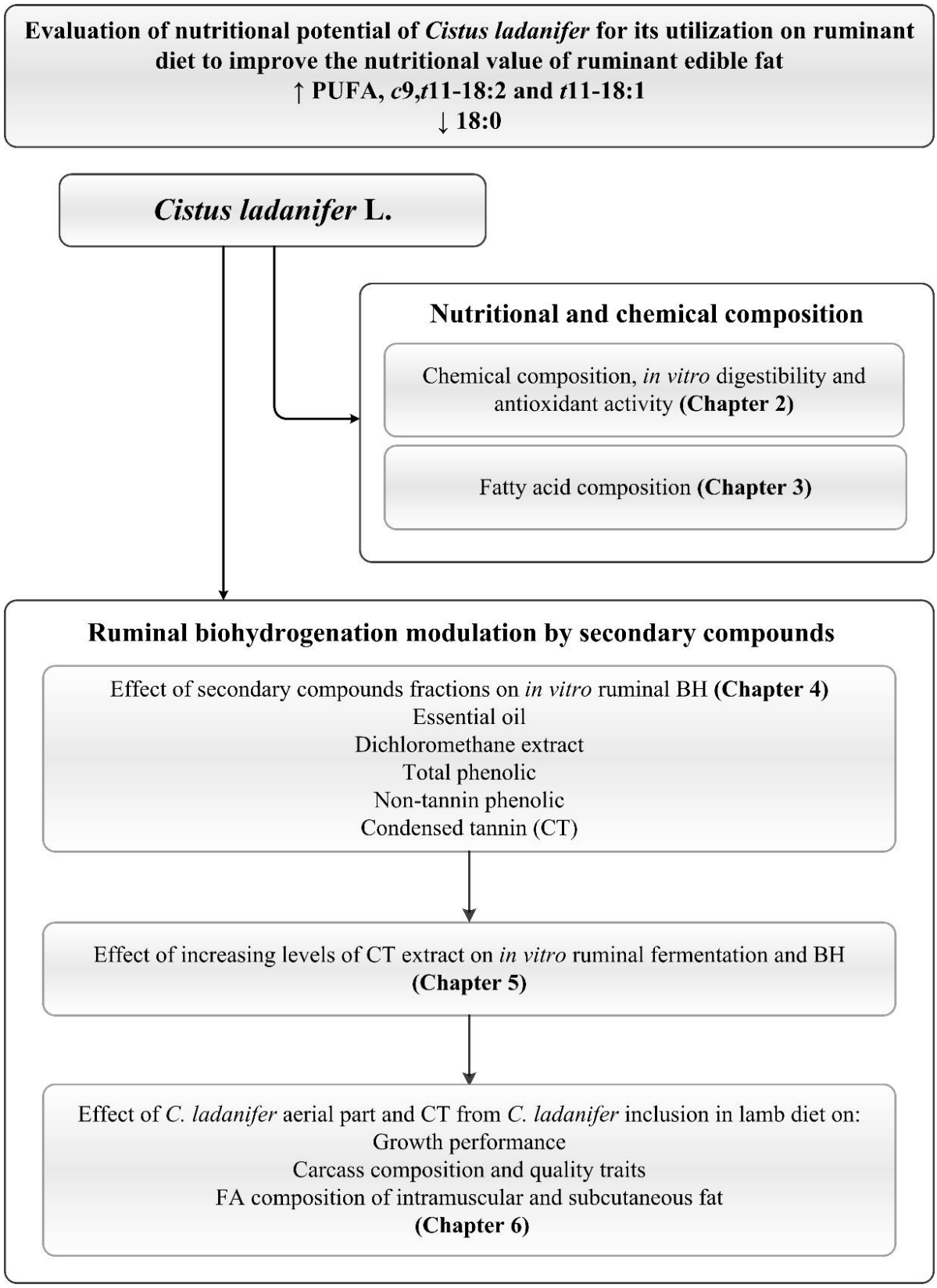
## 1. 5 Research objectives

*Cistus ladanifer* is a shrub quite abundant in the Mediterranean countries, which is considered an unbalanced feed with poor nutritional value due to high CT content. However, it can be considered as supplement of other feeding resources that complements its nutritional imbalances. *Cistus ladanifer* when incorporated in oil supplemented lamb diets improved the beneficial FA contents in intramuscular fat, without compromise the animal productive performance. Nevertheless, *C. ladanifer* is composed by several secondary metabolites, and the information about its chemical and nutritional composition is still very scarce, as it is not clear which *C. ladanifer* fraction might be responsible for the BH modulation. Therefore, the general objective of this work is to further knowledge regarding *C. ladanifer* nutritional composition for utilization on ruminant diets and to elucidate the mechanisms by which *C. ladanifer* extracts modulate the ruminal BH and improve the nutritional value of ruminant products. In order to achieve this goal, this study involved two main objectives (Figure 1.10):

- To determine the nutritional potential of *C. ladanifer* as a component of ruminant diets:
  - To evaluate the chemical composition, *in vitro* digestibility and antioxidant activity of aerial parts of *C. ladanifer* with different ages, throughout a full year (Chapter 2);
  - To clarify the fatty acid composition of *C. ladanifer* aerial parts with different plant ages, throughout a full year (Chapter 3);

- To evaluate the effect of *C. ladanifer* secondary compounds on BH modulation, particularly the accumulation of *c*9,*t*11-18:2 and *t*11-18:1 in the rumen (*in vitro* and *in vivo* experiments):
  - To clarify which *C. ladanifer* group of secondary compounds are responsible for rumen BH effects (Chapter 4);
  - To determine the amount of *C. ladanifer* CT that optimize the *c*9,*t*11-18:2 and *t*11-18:1 production, by evaluating the effect of increase doses of *C. ladanifer* CT on *in vitro* fermentation and BH (Chapter 5);
  - To investigate the effect of *C. ladanifer* aerial part, and CT fraction extracted from *C. ladanifer* inclusion in lamb diets on growth performance, carcass composition and quality traits and fatty acid composition of intramuscular and subcutaneous fat (Chapter 6).

**Figure 1.10.** Schematization of the main objectives of the present work



# CHAPTER 2

## POTENTIAL OF *CISTUS LADANIFER* L. (ROCKROSE) IN SMALL RUMINANT DIETS – EFFECT OF SEASON AND PLANT AGE ON CHEMICAL COMPOSITION, *IN VITRO* DIGESTIBILITY AND ANTIOXIDANT ACTIVITY

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Adapted from *Grass and Forage Science* (2016), 71, 437-447

(Available online at http://dx.doi.org/10.1111/gfs.12188)

## Abstract

Shrubs can provide an important contribution to the fodder resources for small ruminants in Mediterranean areas, but there is limited information on their feed value, including secondary metabolites and their seasonal differences. This study evaluated the effect of seasonal variation in chemical composition, *in vitro* digestibility and antioxidant activity of the aerial parts of plants of *Cistus ladanifer* of two age groups [young plants *vs*. older ones (2–6 years old)]. Aerial parts of *C. ladanifer* plants of both age groups were characterized by moderate cell wall content (321–410 g NDF/kg DM), high levels of phenolic compounds (551–106 g gallic acid equivalents/kg DM) and condensed tannins (CT) (321–161 g/kg DM), low protein content (55–100 g/kg DM) and low digestibility (249–315 g of digestible organic matter/kg DM). During autumn and winter, *C. ladanifer* showed higher protein levels and lower cell wall content than in the other seasons. The highest values of phenolic compounds, CT and antioxidant activity were achieved during summer. Young plants showed higher levels of phenolic compounds during spring, summer and winter, and higher CT contents in summer (more 54 g/kg DM) compared to old plants. Aerial parts of *C. ladanifer* of both age groups may be used as components of ruminant nutrition, but only as a supplement and associated with other feeding resources to complement its nutritional imbalances.

**Keywords*:*** *Cistus ladanifer*, seasonal variation, plant age, chemical composition, digestibility, antioxidant activity

## 2. 1 Introduction

Shrubs play an important role as browse species in small-ruminant feeding systems in Mediterranean area, providing energy and nutrients during season of pasture scarcity (Bruno-Soares *et al.*, 2011). The rearing of small ruminants in Mediterranean grassland and rangeland areas is limited by seasonal feed deficits, particularly during the summer drought period, which may require expensive feed supplementation. This issue is of increasing relevance in the context of future climate changes, which has focused increased attention on the adaptation of Mediterranean grazing systems to minimize the effects of the expected increased drought (Cosentino, Gresta, & Testa, 2014). Mediterranean areas are highly biodiverse, with a wide range of browse species that have supported livestock over many millennia (Blondel, 2006). The use of shrubs for replacement of part of the cereal concentrate in animal diets, particularly in small ruminants, has been explored in the context of reducing the production cost of livestock feeding (Vasta & Luciano, 2011). The use of shrubs as alternative feed sources may have positive or negative effects. They can be rich sources of secondary metabolites, which due to their recognized bioactivity may be beneficial for animal health, and quality of their derived products, or they may limit their utilization as animal feed. The results of inclusion of plants and plants extracts that are rich in polyphenolic compounds in animal diets have been promising, reporting improvements in oxidative and microbiological stability, fatty acid composition, and organoleptic properties of livestock products (Nieto, Diaz, Banon, & Garrido, 2010; Vasta & Luciano, 2011). Although the nutrient value of some browse species has been evaluated (Ammar, Lopez, Gonzalez, & Ranilla, 2004; Bruno-Soares *et al.*, 2011; Salem, Salem, El-Adawy, & Robinson, 2006; Tolera, Khazaal, & Ørskov, 1997) there are several other potential browse species for which there is only limited information on their potential for use in small-ruminant diets. These might have potential to supplement other feeds, or to provide bioactive compounds as part of nutritional strategies to improve the quality of animal products.

*Cistus ladanifer* L. (rockrose) is a perennial shrub which is widespread in marginal grazing lands in Mediterranean countries (Dentinho *et al.*, 2007). *Cistus ladanifer* is considered an unbalanced feed with poor nutritional value, showing high levels of antinutritional factors, as CT (Dentinho *et al.*, 2005). On the other hand, *C. ladanifer* may represent an important source of bioactive compounds for small ruminant diets, as it contains high levels of different phenolic compounds as phenolic acids and several flavonoids other than CT, and also terpenic compounds (Barrajon-Catalan *et al.*, 2010; Barros *et al.*, 2013; Chaves *et al.*, 1997a; Gomes *et al.*, 2005). Previous results from our research team showed that incorporation of *C. ladanifer* in lamb diets enhances the antioxidant potential of meat, without compromising animal

performance and meat sensory properties (Jerónimo *et al.*, 2012; Jerónimo *et al.*, 2010). Moreover, inclusion of *C. ladanifer* in oil-supplemented diets improved the nutritional value of lamb meat lipid fraction, by increasing its content in rumenic acid (Jerónimo *et al.*, 2010), a conjugated linoleic acid (CLA) which has shown numerous beneficial health properties (Dilzer & Park, 2012). These results provide a basis for encouraging the utilization of *C. ladanifer* in ruminant diets, improving the oxidative stability and fatty acid profile of their derived products. However, there remains a need for information on the nutritional value of *C. ladanifer* for its use as a component of ruminant diets.

Although, it is well known that there are seasonal variations in the nutritional value of shrub species, as well as in their secondary metabolites profile (Ammar *et al.*, 2004; Bruno-Soares *et al.*, 2011; Sfougaris, Nastis, & Papageorgiou, 1996), this information is not known for *C. ladanifer*. To the authors' knowledge, the effect of seasonal variation on chemical composition of *C. ladanifer* is limited to its exudates (Chaves *et al.*, 1997a; Sosa *et al.*, 2005), and to a single study on the chemical composition and digestibility of *C. ladanifer* collected only in two consecutive seasons (Dentinho *et al.*, 2005). Areas occupied by *C. ladanifer* shrubs commonly present plants that persist for several years, as well as new plants that emerge each year. However, the relationship between plant age and nutritional value of *C. ladanifer* is also not known.

Therefore, the objective of this study was to evaluate the chemical composition, including total phenols and CT contents, *in vitro* digestibility and antioxidant activity of aerial parts of *C. ladanifer* with different ages throughout a full year, to assess the nutritional potential of these plants as components of ruminant diets, particularly in nutritional strategies for improving animal health and the quality of their derived products.

## 2. 2 Materials and methods

### 2.2.1 Plant material sampling

*Cistus ladanifer* shrubs were harvested in Baixo Alentejo region, in Monte do Vento, Mértola, Southern Portugal (37°48′N, 7°40′W; 150 m a.s.l.), in a parcel of land containing holm oak *Quercus rotundifolia* L. forest, where *C. ladanifer* plants are the predominant vegetation. There were used two groups of *C. ladanifer* sampled, which consisted of the aerial parts of *C. ladanifer* plants: (i) plants that emerged during current growth season (referred to here as 'young plants'); and (ii) plants with between 2 and 6 growth seasons (i.e. plants 2-6 years old; referred to here as 'old plants'). Samples of both groups were randomly collected during 12

months, between March 2011 and February 2012, with three collections in each season [spring (March, April and May), summer (June, July, and August), autumn (September, October and November) and winter (December, January and February)], with about 1 month between each sampling. Samples were manually harvested with scissors and pooled into three samples. Immediately after collection, plant material was transported to the laboratory and kept frozen at −20 °C until processing. The plant material was then dried in a ventilated oven at 65 °C until constant weight, and ground using a mill to pass a 1-mm sieve. The collected aerial part of plants consisted of leaves, soft stems, and reproductive organs (flower buds, flowers and seed heads). The *C. ladanifer* shrubs showed leaves during the entire year. Flower bud formation began at the end of winter, and flowering occurred between March and April. *Cistus ladanifer* plants with closed seed heads were observed from May to November.

### 2.2.2 Chemical analyses

Ground samples (1 mm) of *C. ladanifer* were analysed for dry matter (DM) (ISO 6495, 1999b), ash (AOAC, 1990 #942.05) and Kjeldahl N (AOAC, 1990 #954.01). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) was determined according to the procedure described by Goering and Van Soest (1970). Neutral detergent fibre was assayed with sodium sulphite, without a heat stable amylase and expressed with residual ash. Acid detergent fibre also is expressed inclusive of residual ash. Gross energy (GE) was measured by calorimetric method (ISO 9831, 1998), and the ether extract (EE) was determined by Soxhlet extraction with petroleum ether (ISO 6492, 1999a). *In vitro* organic matter digestibility (IVOMD) was assayed by the Tilley and Terry method modified by Alexander and McGowan (1966). The minerals Ca, Na, K and Mg were determined by atomic absorption spectrometry (ISO 6869, 2000) and P by UV/vis spectrometry (ISO 6491, 1995). The samples were analyzed in duplicates.

### 2.2.3 Extract preparation for determination of phenolic contents and antioxidant activity

Ground samples (10 g with 3-4 mm) of *Cistus ladanifer* were extracted with 50 mL of acetone solution (70%, v/v) at room temperature (RT) during 2 h, with gentle agitation (800 *g*). Extracts were then centrifuged at 10 000 *g* for 15 min, and the supernatants collected. The leftover residue was further washed with 40 mL of acetone solution (70%, v/v), and centrifuged at the

same conditions as described above. Both supernatants were pooled and then filtered through a 5- to 13-µm filter paper. Extracts were storage at -20°C until further analysis.

#### 2.2.3.1 Determination of total phenolic and condensed tannins contents

Total phenolic (TP) content was quantified using the Folin-Ciocalteu assay, described by Falleh *et al.* (2008) with some modifications. Briefly, aliquots of acetone extract (100 µL) were mixed with 1.5 mL of Folin-Ciocalteu reagent (1:10 v/v) (Merck, Darmstadt, Germany) and left for 5 min at RT protected from light. Then, 1.5 mL of sodium carbonate (60 g/L) (Merck, Darmstadt, Germany) was added, and the mixture was incubated, in the dark, at RT for 1h. The absorbance was read at 725 nm in a double-beam UV/vis scanning spectrophotometer (Helios alpha spectrophotometer; Thermo Scientific, Bremen, Germany). The concentration of TP was measured as gallic acid equivalent using gallic acid (Sigma Chemicals Co., Madrid, Spain) as standard.

Condensed tannins were determined using butanol-HCl method, according to the procedure described by Porter et al. (1986). Briefly, 1 mL of acetone extract was added to 6 mL of acid butanol [5% (v/v) of hydrochloric acid in *n*-butanol] and 0.2 mL of ferric reagent [2% (w/v) of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2M HCl] (Sigma Chemicals Co.). After 50 min of reaction, at 100 ºC in a water bath, samples were chilled, and absorbance was recorded at 550 nm using a double-beam UV/vis scanning spectrophotometer. The concentration of CT was quantified using *C. ladanifer* purified CT as standard, which was purified using Sephadex LH-20 (GE Healthcare Bio-Science, Uppsala, Sweden) chromatographic column, according to Strumeyer and Malin (1975). In each sample, the contents of TP and CT were analyzed in triplicate.

#### 2.2.3.2 Evaluation of antioxidant activity

The antioxidant activity was evaluated by assessing the scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power assay. For determination of antioxidant activity, powder extract was used; this was obtained by sequential removal of acetone, by rotator evaporation at 30 ºC (VV2000; Heidolph Instruments GmbH, Schwaback, Germany), and the water removal by freeze-drying (FTS Systems, Inc., Stone Ridge, NY, USA).

DPPH scavenging capacity was assessed according to the method of Hatano *et al.* (1988). Samples (0.3 mL) dissolved in distilled water, containing several extract concentrations (0.1-100 µg/mL), were mixed with 1.08 x $10^{-8}$ mol of DPPH (Sigma Chemicals Co.) methanolic

solution. The mixture was vortexed and left to stand, in the dark for 1 h. Absorbance of DPPH free radicals was measured at 517 nm against a blank constituted by 3 mL of solvent (distilled water plus methanol) in a double-beam UV/vis scanning spectrophotometer. Scavenging effect was calculated as percentage of DPPH discoloration using the equation: % scavenging effect = [(ADPPH – AS)/ADPPH] x 100, where AS is the sample absorbance and ADPPH is the DPPH solution absorbance. The concentration (mg extract per mililitre) providing 50% DPPH neutralization effect ($EC_{50}$) was calculated from the graph of scavenging effect percentage against the concentration logarithm. The EC50 expressed in mg extract per mililitre was converted into mg extract per kg DM, considering the extraction yield, the amount of *C. ladanifer* (g DM) needed for providing 50% DPPH neutralization was further calculated.

The reducing power was determined according to the method of Oyaizu (1986). Samples (2.0 mL), in distilled water, containing different extract concentrations (0.1-50 μg/mL) were mixed with 2.0 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.0 mL of 1% (w/v) potassium ferricyanidine (Sigma Chemicals Co.). The mixture was incubated at 50 ºC for 20 min. The reaction was stopped by addition of 2.0 mL of 10% (w/v) trichloroacetic acid (BDH Prolabo International, Nogent sur Mance, France) solution. The mixture was further shaken and centrifuged at 6000 *g* for 10 min. The upper layer (1.0 mL) was added to 1.0 mL of distilled water and 2.0 mL of 0.1% (w/v) ferric chloride (III) solution (Sigma Chemicals Co.). The mixture was vortexed and the absorbance of Prussian blue read at 700 nm in a double-beam UV/vis scanning spectrophotometer. Concentration (mg extract per mililitre) providing 0.500 of absorbance ($EC_{50}$) was calculated from the absorbance graph plotted at 700 nm against concentration. The EC50 expressed in mg extract per mililitre was converted into mg extract per kg DM. The amount of *C. ladanifer* (g DM) needed for providing 50% reduction of the $Fe^{3+}$/ferricyanidine complex to the ferrous form was further calculated, considering the extraction yield. The scavenging capacity of DPPH and reducing power was analyzed in triplicate for each sample.

### 2.2.4 Statistical analysis

All parameters measured were analyzed using the PROC GLM option of SAS (SAS Institute Inc., Cary, NC). Data were analyzed using a completely randomized design with four seasons (spring, summer, autumn and winter) × 2 plant age groups (*C. ladanifer* plants from current growth season and *C. ladanifer* plants with 2-6 growth seasons) in a factorial arrangement with three repetitions (pooled samples). Least square means and standard error of the mean are

presented in tables, except for antioxidant activity, which least square means and standard deviation are present in figures.

## 2. 3 Results

### 2.3.1 Proximate composition, phenolic contents and *in vitro* digestibility

The chemical composition, contents of TP and CT, and IVOMD of aerial parts of *C. ladanifer* are present in Table 2.1. The DM content of *C. ladanifer* varied throughout the seasons ($P < 0.001$), ranging from 415 g/kg in spring to 526 g/kg in summer, with intermediate values during autumn and winter (460 g/kg). In addition to seasonal variation, the DM content tended to be higher ($P = 0.051$) in old *C. ladanifer* plants than young ones (481 *vs*. 449 g/kg DM).

Significant interactions between season and plant age were found for ash content ($P = 0.009$), CP, NDF, ADF ($P < 0.001$), ADL ($P = 0.004$) and EE ($P = 0.028$). The ash content of *C. ladanifer* 'old plants' remained unchanged throughout the year, averaging 41.7 g/kg DM, while in 'young plants', the ash content decreased from 44.7 g/kg DM in winter and spring to 38.2 g/kg DM in summer and autumn. During all seasons, the ash level was similar among both plant groups, except in summer where 'young plants' showed lower values than 'old plants'.

The aerial part of *C. ladanifer* of both plant groups showed highest CP content during winter (100 and 90.8 g/kg DM in young and in old plants, respectively). In young *C. ladanifer* plants, the CP level decreased by 39.9 g/kg DM between winter and spring, remaining constant during spring and summer (57.6 g/kg DM) and increased in autumn for intermediate values. In 'old plants', CP content decreased by 34.4 g/kg DM between winter and summer and increase in autumn for values found in spring (65.5 g/kg DM). The CP levels did not differ among plant groups throughout the year, except in autumn where aerial parts of 'young plants' had more 17 g/kg DM of CP than those from older plants.

During spring, summer and autumn, the EE content of *C. ladanifer* aerial part was similar among plant groups, remaining unchanged during spring and summer (59.4 g/kg DM) and increasing in autumn (79.5 g/kg DM). The EE content increased in aerial part of 'old plants' up to a maximum level in winter (90.7 g/kg DM), whereas in 'young plants', the EE content remained constant. During winter, 'old plants' showed more 13.4 g/kg DM of EE than 'young plants'.

**Table 2.1.** Effect of season and plant age on chemical composition and *in vitro* organic matter digestibility of *Cistus ladanifer*.

| | Spring | | Summer | | Autumn | | Winter | | s.e.m. | *P* values | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Young | Old | Young | Old | Young | Old | Young | Old | | Season (S) | Age (A) | S×A |
| DM[1] | 396 | 435 | 527 | 524 | 467 | 503 | 407 | 464 | 29.1 | <0.001 | 0.051 | 0.562 |
| Ash[2] | 44.7$^{a}$ | 41.8$^{ab}$ | 37.3$^{c}$ | 42.3$^{ab}$ | 39.0$^{bc}$ | 40.4$^{bc}$ | 44.6$^{a}$ | 42.4$^{ab}$ | 0.71 | 0.002 | 0.712 | 0.009 |
| CP[2] | 60.1$^{cd}$ | 67.1$^{c}$ | 55.0$^{d}$ | 56.4$^{d}$ | 82.9$^{b}$ | 65.9$^{c}$ | 100$^{a}$ | 90.8$^{ab}$ | 3.37 | <0.001 | 0.058 | <0.001 |
| EE[2] | 57.1$^{c}$ | 58.0$^{c}$ | 62.9$^{c}$ | 61.2$^{c}$ | 81.4$^{b}$ | 77.5$^{b}$ | 77.3$^{b}$ | 90.7$^{a}$ | 3.12 | <0.001 | 0.307 | 0.028 |
| NDF[2] | 381$^{bcd}$ | 395$^{ab}$ | 410$^{a}$ | 389$^{bc}$ | 361$^{e}$ | 365$^{de}$ | 321$^{f}$ | 374$^{cde}$ | 6.8 | <0.001 | 0.013 | <0.001 |
| ADF[2] | 283$^{cd}$ | 297$^{bcd}$ | 339$^{a}$ | 313$^{b}$ | 304$^{bc}$ | 278$^{d}$ | 277$^{d}$ | 316$^{b}$ | 7.4 | <0.001 | 0.921 | <0.001 |
| ADL[2] | 85.6$^{e}$ | 97.0$^{d}$ | 117$^{ab}$ | 121$^{ab}$ | 123$^{ab}$ | 112$^{bc}$ | 104$^{cd}$ | 125$^{a}$ | 4.64 | <0.001 | 0.037 | 0.004 |
| TP[3] | 98.1$^{ab}$ | 74.4$^{d}$ | 106$^{a}$ | 87.4$^{bc}$ | 65.5$^{de}$ | 60.3$^{e}$ | 85.9$^{c}$ | 55.1$^{e}$ | 3.91 | <0.001 | <0.001 | 0.014 |
| CT[2,4] | 53.5$^{c}$ | 39.7$^{c}$ | 161$^{a}$ | 107$^{b}$ | 99.6$^{b}$ | 83.6$^{b}$ | 32.1$^{c}$ | 52.6 | 8.31 | <0.001 | 0.009 | <0.001 |
| Minerals[2] | | | | | | | | | | | | |
| Ca | 7.2 | 7.1 | 5.9 | 6.3 | 4.9 | 4.7 | 6.1 | 4.8 | 0.44 | <0.001 | 0.273 | 0.159 |
| P | 1.6 | 1.3 | 1.5 | 1.5 | 1.5 | 1.4 | 2.1 | 2.0 | 0.07 | <0.001 | 0.011 | 0.122 |
| Na | 0.28 | 0.63 | 0.18 | 0.34 | 0.39 | 0.65 | 0.57 | 0.58 | 0.088 | 0.003 | 0.003 | 0.272 |
| K | 5.4 | 4.2 | 4.3 | 3.9 | 3.5 | 3.7 | 5.1 | 4.4 | 0.48 | 0.015 | 0.102 | 0.444 |
| Mg | 2.1 | 2.1 | 2.5 | 2.5 | 2.8 | 2.4 | 1.9 | 1.9 | 0.23 | 0.010 | 0.323 | 0.478 |
| GE[5] | 20.0 | 20.2 | 20.4 | 20.4 | 21.2 | 21.0 | 20.3 | 20.7 | 0.22 | <0.001 | 0.432 | 0.627 |
| IVOMD[2] | 297$^{a}$ | 299$^{a}$ | 261$^{b}$ | 299$^{a}$ | 249$^{b}$ | 315$^{a}$ | 311$^{a}$ | 310$^{a}$ | 10.7 | 0.008 | <0.001 | 0.003 |

Young, plants that emerged during current growth season; Old, plants with between 2 and 6 growth season; DM, dry matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; TP, total phenolic; CT, condensed tannins; GE, gross energy; IVOMD, *in vitro* organic matter digestibility; s.e.m., standard error of the mean. [1] g/kg; [2] g/kg DM; [3] g gallic acid equivalents/kg DM; [4] CT - quantified using purified *Cistus ladanifer* CT as standard; [5] MJ/kg DM; means with different letters within the same row are statistically different ($P < 0.05$).

The cell wall content (Table 2.1) of the aerial parts of *C. ladanifer* 'young plants' was more variable throughout the seasons than in older ones, showing an additional 89 g/kg DM of NDF during summer than in the winter season, while in older plants, the NDF content increased only by 22 g/kg DM from autumn/winter to spring/summer. In spring and autumn, both plant age groups had similar NDF contents: 388 and 363 g/kg DM respectively. However, there were differences during the rest of the year, and aerial parts of 'young plants' had 21 g/kg more NDF in summer and 53 g/kg less NDF in winter, compared to older ones.

The hemicellulose content (data not shown, calculated as the difference between NDF and ADF) of *C. ladanifer* aerial parts was only affected by season ($P < 0.001$), ranging from 50.7 g/kg DM in winter to 97.7 g/kg DM in spring, while plants collected during summer and autumn had intermediate hemicellulose values (74.8 g/kg DM). For the cellulose content (data not shown, calculated as the difference between ADF and ADL), an interaction between season and plant age was observed ($P < 0.001$). In 'young plants', the cellulose content ranged from 176 g/kg DM in autumn and winter to 222 g/kg DM in summer, while in spring, there was an intermediate cellulose content (196 g/kg DM). On the other hand, the cellulose content in 'old plants' had less fluctuation, ranging from 162 g/kg DM in autumn to 194 g/kg DM in other seasons. In summer, 'young plants' had 28 g/kg DM more cellulose than 'old plants', whereas during other seasons, the cellulose content did not differ among plant age groups.

Both of the *C. ladanifer* age groups showed lower ADL content (lignin) in spring (Table 2.1), compared to the other seasons. 'Young plants' showed highest lignin content during summer and autumn (120 g/kg DM); this value decreased to 34.4 g/kg DM by spring. However, in 'old plants', the lignin values remained relatively constant between summer and winter (112-125 g/kg DM).

The GE content of *C. ladanifer* aerial parts was only affected by season ($P < 0.001$), ranging from 20.1 MJ/kg DM in spring, to 21.1 MJ/kg DM in autumn, while plants collected during summer and winter had intermediate GE contents (20.5 MJ/kg DM).

Significant interactions between season and plant age were found for contents of TP ($P = 0.014$) and CT ($P < 0.001$) (Table 2.1). In young *C. ladanifer* plants, the TP content ranged from 65.5 g/kg DM in autumn to 102 g/kg DM during summer and spring, whereas plants collected in winter showed intermediate TP content (85.9 g/kg DM). In 'old plants' the lowest TP values were observed during autumn and winter (57.7 g/kg DM), a value that increased during spring up to a maximum level in summer (87.4 g/kg DM). In all seasons, 'young plants' showed higher TP levels than older plants, except during autumn when the TP content was equal among plant groups.

During spring, autumn and winter, the CT content of *C. ladanifer* aerial parts was similar among plant age groups (Table 2.1). In autumn CT was averaged 91.6 g/kg, decreasing to minimum values (44.5 g/kg DM) during winter and spring. The highest CT contents were observed during summer, for both plant age groups, increasing by about 107 and 67 g/kg DM relative to spring in young and older plants respectively. In summer, 'young plants' had 54 g/kg DM more CT than 'old plants'.

For IVOMD, a significant interaction between season and plant age was also observed ($P$ = 0.003). The seasonal variation in IVOMD was observed only in 'young plants', decreasing from 304 g/kg DM in winter and spring to 255 g/kg in summer and autumn, while in 'old plants' IVOMD remained unchanged throughout the seasons and averaged 281 g/kg DM. During summer and autumn, 'young plants' showed lower IVOMD than 'old plants', whereas in other seasons, the IVOMD did not differ between the plant age groups (Table 2.1).

### 2.3.2 Mineral composition

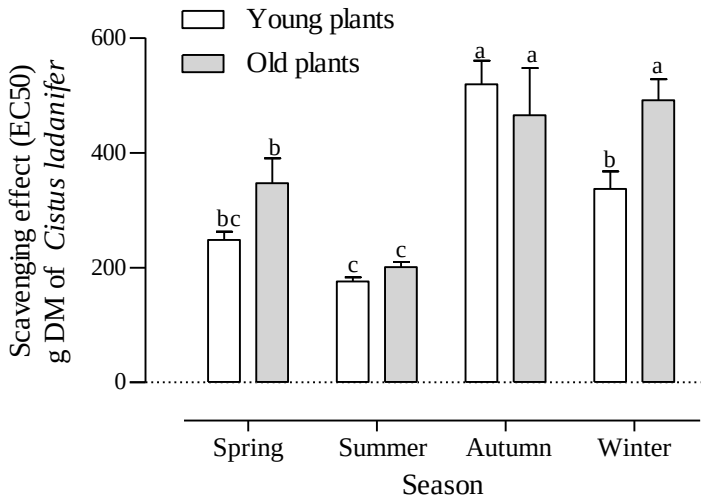
According to the results presented in Table 2.1, the contents of Ca, K and Mg of *C. ladanifer* plants were affected only by season. The Ca content of *C. ladanifer* aerial parts ranged from 4.8 g/kg DM in autumn to 7.1 g/kg DM in spring, while in summer and winter, intermediate values were observed, between autumn and spring (5.8 g/kg DM). *Cistus ladanifer* plants harvested during winter and spring showed higher K content than those harvested in autumn (4.8 *vs*. 3.6 g/kg DM), while during summer, the K content was similar to the observed in other seasons. The Mg content increased from 1.9 g/kg DM in winter to 2.5 g/kg DM in summer, and during spring and autumn the Mg content was equal to that observed in summer and winter.

The P and Na contents (Table 2.1) of *C. ladanifer* aerial parts were affected by both season and plant age. Aerial parts of 'young plants' showed higher P content and lower Na content than 'old plants' (1.7 *vs.* 1.6 g/kg DM of P and 0.36 *vs.* 0.56 g/kg DM of Na in young and older plants, respectively). *Cistus ladanifer* plants harvested in summer had lower Na content (0.25 g/kg DM) than those harvested in other seasons (0.52 g/kg DM). The P content remained constant during spring, summer and autumn, averaging 1.5 g/kg DM, and increasing for 2.0 g/kg DM in winter.

### 2.3.3 Antioxidant activity

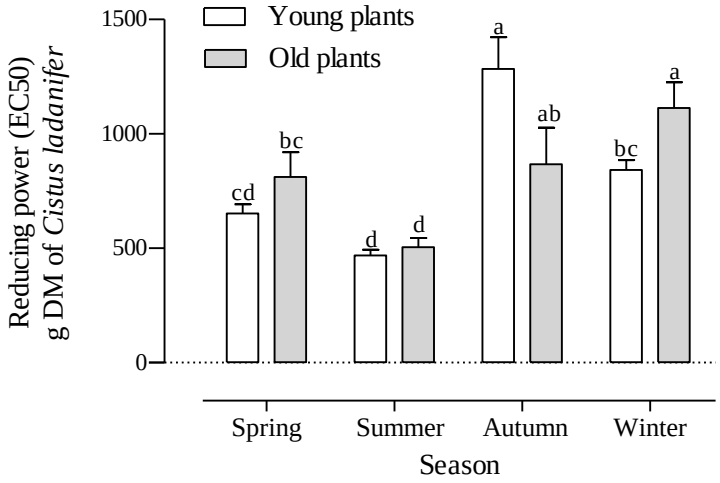
The variation of scavenging capacity and reducing power of *C. ladanifer* throughout the seasons are presented in Figure 2.1 and 2.2 respectively. The lower $EC_{50}$ implies a higher scavenging capacity or reduction power. Significant interactions between season and plant age were found for scavenging capacity ($P = 0.039$) and reducing power ($P = 0.010$). The scavenging capacity and reducing power in each of the *C. ladanifer* groups followed the same trend throughout the seasons. 'Young plants' presented the lowest antioxidant activity in autumn, increasing progressively between winter and summer. On the other hand, 'old plants' carried the lowest scavenging capacity and reducing power during autumn and winter, increasing in spring to up maximum values in summer. The antioxidant activity of plants in each of the two age groups was similar throughout the year, except during winter where the 'young plants' showed higher antioxidant activity than old ones.

**Figure 2.1.** Effect of season and plant age on scavenging effect (EC50, g DM) of *Cistus ladanifer*




Young plants, plants that emerged during current growth season; Old plants, plants with between 2 and 6 growth season; Values are means, with standard deviation represented by vertical bars. Values with different superscripts are significantly different ($P < 0.05$).

**Figure 2.2.** Effect of season and plant age on reducing power (EC50, g DM) of *Cistus ladanifer*.




'Young plants', plants that emerged during current growth season; 'Old plants', plants with between 2 and 6 growth season; Values are means, with standard deviation represented by vertical bars. Values with different superscripts are significantly different ($P < 0.05$).

## 2. 4 Discussion

Aerial parts of plants *C. ladanifer*, and each of two age groups, are characterized by moderate NDF content (321-410 g/kg DM), lower contents of CP (55-100 g/kg DM) and low digestibility (249-315 g/kg DM), in agreement with previously reported results for *C. ladanifer* (Dentinho *et al.*, 2005), as well as for other *Cistus* species (Sfougaris *et al.*, 1996). The EE content of aerial parts of *C. ladanifer* ranged from 57.1 to 90.7 g/kg DM, and values of about 9.3 g/kg DM of EE were also reported in aerial part of *C. ladanifer* by Dentinho et al. (2005). However, recent results have shown that the total fatty acid content of *C. ladanifer* aerial part varies from 5.0 to 9.0 mg/kg DM, only about 10% of the EE (Guerreiro, Alves, Duarte, Bessa, & Jerónimo, 2015), 2015). In the present study, the aerial parts of *C. ladanifer* comprised a mixture of leaves, soft stems and reproductive organs. A comparative analysis between our results and data reported for other *Cistus* shrubs is difficult, because in most previous studies on the nutritive value of *Cistus* species, there was separate evaluation of the different plant parts (leaves, flowers, flower buds and seed heads) instead of analysis of the mixture of such plant parts (Ammar *et al.*, 2004; Bruno-Soares, Ferreira, Sousa, & Abreu, 1999; Bruno-Soares *et al.*, 2011).

As expected, the chemical composition of aerial parts of *C. ladanifer* varied throughout the seasons, and this is closely related to the plant vegetative stage. The phenology of *C. ladanifer* has been described (Cabezudo *et al.*, 1992; Talavera *et al.*, 1993); however, the timing and duration of each phase of vegetative development is not exactly equal, and this may be related to the year and place of growth. In general, it is considered that the vegetative growth of *C.*

*ladanifer* starts after the first autumn rains (with emergence of new plants, as well as regrowth of new leaves in existing plants that developed in previous seasons), after being reduced during the summer dry season (Talavera *et al.*, 1993). Formation of flower buds starts during winter and flowering occurs in late winter and spring (Cabezudo *et al.*, 1992; Talavera *et al.*, 1993). During summer, the seed heads open, and seed dispersal occurs by early, which might lead to establishment of new plants (Talavera *et al.*, 1993). Leaf drop occurs all year (Cabezudo *et al.*, 1992).

In accordance with the phenological cycle, *C. ladanifer* showed relatively high CP and low NDF contents during autumn and winter (in early stages of the growth season), while during spring and summer, there was reduction in CP and increase in the NDF content, as result of the plant maturation. During the vegetative development, reduction in CP content and increase in the fibre fraction in the aerial parts of other *Cistus* species have been reported previously (Sfougaris *et al.*, 1996). In addition, reduction in CP content in leaves of *Cistus salviifolius* has been observed during vegetative development (Bruno-Soares *et al.*, 1999; 2011).

Regardless of the plant age, lignin content increased between spring and summer in aerial parts of *C. ladanifer* plants. However, this increase was only of 29.7 and 22 g/kg DM, respectively, in young and old plants, whereas Sfougaris *et al.* (1996) reported an increase of 77 g/kg DM in aerial parts of other *Cistus* species for the same seasons. Moreover, for 'young plants', the lignin value did not change between summer and autumn, while in the old plants group, the lignin content maintained relatively unchanged during summer, autumn and winter. Conversely, Sfougaris *et al.* (1996) observed lower lignin content in *Cistus* species harvested during autumn and winter, than those harvested in summer. These differences are probably related to environmental factors (temperature, water availability and soil fertilization), which are known to affect cell-wall lignification (Van Soest, 1994), contributing for distinct lignin contents.

The IVOMD was lower in 'young plants', especially during summer and autumn, while the IVOMD in 'old plants' remained unchanged throughout the year, in accordance with Sfougaris *et al.* (1996) who also reported a constant IVOMD over the seasons for other *Cistus* shrubs. The lignin content is considered to be the principal factor limiting digestibility, but other plant components involved in plant self-protection can also limit the nutrients digestibility, such as CT (Van Soest, 1994). The CT content of *C. ladanifer* ranged from 3.2 to 16.1% of DM, being particularly high during summer. Condensed tannins can complex with numerous types of molecules, primarily with proteins and to a lesser extent with polysaccharides or amino acids, conditioning the digestible utilization of these nutrients, particularly the protein (Makkar *et al.*, 2007a). Therefore, excessive intake of CT might be detrimental; however, the dietary CT might

have benefits or detrimental effects in ruminants, depending on the chemical structure and concentration in diets, the composition of basal diet and on the other factors intrinsically related to the animals, such as species and physiological stage (Makkar *et al.*, 2007a; Piluzza *et al.*, 2014; Waghorn, 2008). Low or moderate intake of CT generally has beneficial effects, preventing the bloat and enhancing the protein utilization during digestion, as well as improved growth performance, wool growth, and milk production and milk protein concentration (Min *et al.*, 2003; Waghorn, 2008). Beneficial effect of CT-containing forages in control of internal parasites also has been reported (Piluzza *et al.*, 2014). Conversely, high CT concentrations may adversely affect dietary preference and are associated with reduced feed intake, digestibility of fibre and nitrogen, and animal performance (Makkar *et al.*, 2007a; Min *et al.*, 2003; Waghorn, 2008). Incorporation of tannin crude extract from *C. ladanifer* in soya bean meal, to obtain diets with CT levels that vary between 0.6 to 7% in DM, is reported to reduce the rumen degradation of protein, as well the protein intestinal digestibility (Dentinho *et al.*, 2014; Dentinho *et al.*, 2007). However, those studies did not evaluate whether this reduction in protein availability has implication for animal performance. As far as we know, the effect of dietary inclusion of *C. ladanifer* on animal growth performance is restricted to two trials with lambs, in which 50, 100, 200 and 250 g/kg DM of the diet consisted of leaves and soft stems of *C. ladanifer* (Francisco *et al.*, 2015; Jerónimo *et al.*, 2012; Jerónimo *et al.*, 2010). In both these studies, the feed intake and animal performance was not affected by the dietary incorporation of *C. ladanifer* (Francisco *et al.*, 2015; Jerónimo *et al.*, 2010), even in trial with highest levels of *C. ladanifer* (250 g/kg DM), which resulted in diets with about 21 g/kg DM of CT (Jerónimo *et al.*, 2010). The IVOMD results for *C. ladanifer* obtained in present study should be interpreted with caution, once the *in vitro* methodology used for its quantification may not be suitable for evaluating feeds with antinutritive factors and for predicting animal performance. The gas production technique seems to be more efficient for determining the nutritional value of feeds containing antinutritive factors, allowing the evaluation of the fermentation evolution and the possible interactions with the microbial population and with other nutrients, unlike the Tilley and Terry method which only quantifies the residues remaining after fermentation (Khazaal & Ørskov, 1994; Makkar, 2005).

Plant material of *C. ladanifer* from both plant age groups is characterized by high levels in polyphenolic compounds (55.1-106 g/kg DM), mainly during spring and summer. Polyphenolic compounds are known to have antioxidant activity, and high antioxidant activity of various phenolic extracts from *C. ladanifer* had been reported (Andrade *et al.*, 2009; Barrajon-Catalan *et al.*, 2010). In accordance with total phenol content, the aerial parts of *C. ladanifer* showed the highest antioxidant activity during spring and summer. Synthetic antioxidants, such as

butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), are widely used in animal nutrition. However, consumers are increasingly aware of food safety issues associated with toxicity of synthetic antioxidants, leading to increasing interest in natural antioxidants. Plants rich in polyphenolic compounds have been extensively explored as natural sources of antioxidants in animal nutrition, and incorporation of *C. ladanifer* in lamb diets has been shown to be effective for improving resistance of meat to lipid oxidation (Francisco *et al.*, 2015; Jerónimo *et al.*, 2012).

Variation of the CT and TP contents in aerial parts of *C. ladanifer* throughout the seasons was expected, as its biosynthesis is linked environmental conditions and plant growth stage. High environmental temperatures and hydric stress are associated with high synthesis of polyphenolic compounds, including tannins (Mangan, 1988), resulting in an increase of the CT and TP contents during dry seasons. On the other hand, lower CT content during growing season may be related with allocation of more carbohydrates for growth and reproduction than to production of tannins during this stage of plant growth (Skogsmyr & Fagerstrom, 1992).

The mineral composition of Mediterranean shrubs has been little explored and, to the authors' knowledge, this is the first report on the mineral composition of *C. ladanifer*. Within the site-specific limitations of the data reported here, we can conclude that the mineral composition of *C. ladanifer* was affected mainly by season, with the effect of plant age being limited to P and Na contents (of the nutrients analysed). Seasonal variation was inconsistent among the several mineral elements analyzed: there were high contents of Ca and K during spring, and the highest levels of Mg were reached in summer, whereas the aerial parts of *C. ladanifer* harvested in winter showed the highest Na and P contents. Seasonal variation in mineral composition was been reported previously for other Mediterranean shrubs, including *Cistus* species (Gokkus, Parlak, & Parlak, 2011). However, the seasonal variations reported for *Cistus creticus* L. (Gokkus *et al.*, 2011) are not fully consistent with our results. In grazing production systems, the supply of minerals is frequently dependent on the available feed resources. Extensive ruminant production in Mediterranean areas is mainly based on grazing of natural grasslands in which several shrubs species abound, so the contribution of shrubs to meet the mineral requirements of ruminants should be considered. Taking in account the mineral requirements for ruminants (McDowell & Valle, 2000), the aerial parts of *C. ladanifer* may be a dietary source of Ca and Mg for grazing animals. However, the possible contribution of *C. ladanifer* to supply the needs of ruminant for P, Na and K is relatively low, as the contents of these minerals are low compared with the animals' requirements.

The chemical composition, *in vitro* digestibility and antioxidant activity of aerial parts of *C. ladanifer*, was mainly affected by season; however, effects of plant age, which may modify plant nutritional value and bioactivity, was also observed. The effects of phytochemicals compounds depends on the amount consumed, among many other factors (Acamovic & Brooker, 2005), and it is important to point that 'young plants' showed higher TP content during spring, summer and winter, and higher CT in summer (more 54 g/kg DM), compare to 'old plants'. It is established that secondary compounds may have several roles in plants, but probably their primary use is in plant defence against herbivores, insects and microorganisms, against other plants competing for nutrients and light, and protection against the UV light (Acamovic & Brooker, 2005). Higher production of phytochemical compounds in 'young plants' probably constitutes a defense mechanism that allows their preservation by deterring grazing. It was reported that there was lower intake of phyllodes from young *Acacia* trees by sheep and goats than from old trees, which probably is related to the presence of higher content CT in young *Acacia* phyllodes than in older ones (Degen *et al.*, 1997).

In extensive Mediterranean systems, fibrous feeds, such as silage, hay, and straws and stubbles are important feedstuffs in ruminant diets (Bruno-Soares, Cadima, & Matos, 2010; Bruno-Soares *et al.*, 2011; Cosentino *et al.*, 2014). During autumn and winter, particularly during winter time when relatively low temperatures lead to lower rates of herbage growth on pastures, the aerial parts of *C. ladanifer* showed higher levels of CP and lower cell-wall contents, in comparison with conventional feedstuffs, such as silage, straw and forage crops (Bruno-Soares, Abreu, Guedes, & Dias-da-Silva, 2000; Fonseca, Cabrita, Lage, & Gomes, 2000). Moreover, during periods of low availability of forage, the foliage of several Mediterranean shrubs and trees is also commonly used to supplement the available feed resources. In most cases, the CP content of *C. ladanifer* was higher or similar to values recorded for the foliage of shrubs and tree species such as *Arbutus unedo*, *Pistacia lentiscus*, *Quercus suber* and *Quercus rotundifolia* (Ammar, Lopez, & Gonzalez, 2005; Dentinho *et al.*, 2005). The aerial parts of *C. ladanifer* also had lower cell-wall content compared with foliages of these shrubs and trees, particularly foliages of *Quercus* species that present cell-wall contents above of 500 g/kg DM (Ammar *et al.*, 2005; Dentinho *et al.*, 2005). Although *C. ladanifer* shows some better or similar characteristics to other feed resources usually used by grazing animals, its utilization requires great care. In addition of the possible detrimental effects on animal performance, the occurrence of metabolic disorders cannot be disregarded. Symptoms of toxicity related to intake of *Cistus* species, including *C. ladanifer*, has been reported in grazing ruminants, although this is uncommon (Soler, 2013; Yeruham *et al.*, 2002).

The aerial parts of *C. ladanifer* may potentially be used as an alternative feed resource to supplement diets based on grasslands and concentrates, particularly for small ruminants that show lower energy requirements per animal compared to larger animals, such as cattle. Due to high levels of bioactive compounds found in *C. ladanifer*, its utilization has potential as a component of the diets of small ruminants in the context of nutritional strategies for improving animal health and also for improved quality of livestock food products (Vasta, Nudda, Cannas, Lanza, & Priolo, 2008). However, its high levels of phenolic compounds, particularly CT, throughout the year though mainly in summer, requires great care in ensuring that the quantities incorporated in diets avoid detrimental effects on animal performance and metabolic disorders. Therefore, *C. ladanifer* should to be used only as supplement and associated with other feeding resources that complement its nutritional imbalances.

## Acknowledgements

The authors thank COMPETE (Competitivity Factors Operation Program), QREN (National Strategy Reference, Portugal, 2007–2013) for their financial support to the project "BIOECOS - Valorização Integrada da Biomassa" (ALENT-09-0140-FEDER000705), PRODER (Programa de Desenvolvimento Rural) for their financial support to the project "Rede Temática para a Valorização dos Recursos Silvestres do Mediterrâneo" (020311015068), Fundação para a Ciência e Tecnologia (FCT) through research grants from Olinda Guerreiro (SFRH/BD/84406/2012), Ângela Guerra (SFRH/BD/98635/2013), Patrícia Ramos (SFRH/BD/70845/2010) and Eliana Jerónimo (SFRH/BPD/74527/2010), Associação de Defesa do Património de Mértola (ADPM) for kindly supplying the *Cistus ladanifer L*. samples, and José Batista for cooperation in analytical determinations.

# CHAPTER 3

## *CISTUS LADANIFER* L. SHRUB IS RICH IN SATURATED AND BRANCHED CHAIN FATTY ACIDS AND THEIR CONCENTRATION INCREASES IN THE MEDITERRANEAN DRY SEASON

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Adapted from *Lipids* (2015), 50, 493-501

(Available online at 10.1007/s11745-015-4001-4)

Note: Fatty acids nomenclature are according to the publication in *Lipids*

## Abstract

The *Cistus ladanifer* L. shrub is a widespread species of Mediterranean region that is available as feed source for ruminants all year round, constituting a source of energy and nutrients when most of vegetation is dry. However, there is no trustworthy information about the fatty acid composition of *C. ladanifer*, as well as no information about the seasonal and age related changes on their fatty acid composition. Thus, we collected aerial parts of *C. ladanifer* plants of two age groups [young *vs.* older ones (2-6 years old)] during four consecutive seasons to characterize their fatty acid composition. The fatty acid composition of *C. ladanifer* is dominated by saturated fatty acids including the occurrence of two methyl branched chain fatty acids (BCFA), the iso-19:0 and iso-21:0, which as far as we know were detected for the first time in shrubs. Also, we demonstrate that several labdane type compounds might interfere with the fatty acid analysis of *C. ladanifer*. Marked seasonal changes on BCFA and polyunsaturated fatty acids (PUFA) were found, suggesting that BCFA can replace PUFA in plant lipids at high environment temperatures.

**Keywords:** *Cistus ladanifer*, seasonal variation, plant age, fatty acids, branched chain fatty acids, gas chromatography, mass spectrometry

## 3. 1 Introduction

*Cistus ladanifer* L. shrub, which belongs to the Cistaceae family, is a native and widespread species in marginal fields of Mediterranean region (Barrajon-Catalan *et al.*, 2010; Teixeira *et al.*, 2007). This aromatic shrub forms dense stands on siliceous soils, colonizes degraded areas and inhibits the growth of other plants (Chaves, Sosa, Alias, & Escudero, 2001), showing a high capacity to repopulate after wildfires contributing to their abundance (Ferrandis, Herranz, & Martinez-Sanchez, 1999).

The *C. ladanifer* is available year round, maintaining green leaves even in the Mediterranean summer when most of range vegetation is already dry. Although it is considered as an unbalanced food with poor nutritional value (Dentinho *et al.*, 2005), *C. ladanifer* aerial parts are often used by grazing ruminants, particularly during season of pasture scarcity. Indeed, recent studies have shown that the utilization of aerial parts and condensed tannin extracts from *C. ladanifer* when incorporated into the ruminant diets, improve meat quality, meat resistance against lipid oxidation, modulates rumen biohydrogenation (Jerónimo *et al.*, 2012; Jerónimo *et al.*, 2010) and reduces rumen protein effective degradability (Dentinho *et al.*, 2014); demonstrating the potential of this shrub as an alternative feeding resource in ruminant feeding systems within the Mediterranean area.

The oleoresin that covers the leaves and stalks of *C. ladanifer* (i.e., labdanum) is also used in the perfume industry for its persistence and amber scent (Mariotti *et al.*, 1997). Recently, there has been an increase interest in the bioactive composition and potential of *C. ladanifer* biological activities, being reported as having antioxidant, antibacterial and antifungal activities in its extracts, as well as *C. ladanifer* essential oil (Barrajon-Catalan *et al.*, 2010; Barros *et al.*, 2013). *Cistus ladanifer* contains different types of secondary metabolites, such as phenolic and terpenic compounds, fatty acids and sterols (Barros *et al.*, 2013; Chaves, Escudero, & Gutierrez Merino, 1997b; Gomes *et al.*, 2005). The majority of the studies have been focused on the characterization of the *C. ladanifer* essential oil (Gomes *et al.*, 2005; Verdeguer, Blazquez, & Boira, 2012), and exudates (Chaves *et al.*, 1997b; Sosa *et al.*, 2005), while a few have been focused on phenolic (Barrajon-Catalan *et al.*, 2011; Fernandez-Arroyo, Barrajon-Catalan, Micol, Segura-Carretero, & Fernandez-Gutierrez, 2010) and volatile (Dias & Moreira, 2002; Teixeira *et al.*, 2007) compounds of the aerial parts. As far as we know, only one study attempted to report the fatty acid composition of *C. ladanifer* leaves, presenting a doubtful fatty acid profile in which the identifications were based only by comparison of retention times with commercial standards (Guimarães *et al.*, 2009). Chemical composition of shrubs may vary along seasons and plant age (Ammar *et al.*, 2005; Sfougaris *et al.*, 1996), however there is no

information about these factors on fatty acid composition of *C. ladanifer*. So, the present work aims to clarify the fatty acid composition of *C. ladanifer* aerial parts at different plant ages throughout a full year.

## 3. 2 Material and methods

### 3.2.1 Chemicals

All reagents and solvents were of analytical and chromatographic grade. The nonadecanoic acid methyl ester (19:0), the 17-methylstearic acid (iso-19:0) and the 19-methylarachidic acid (iso-21:0) standards were obtained from Sigma-Aldrich (St. Louis, MO) and were all of highest purity available (GC grade >99%). A 37 component fatty acid methyl esters (FAME) standard mix (Ref. 47885-U) was purchased from Supelco Inc. (Bellefonte, PA, USA).

### 3.2.2 Plant material sampling

*Cistus ladanifer* L. shrubs were harvested between March 2011 and February 2012, in the Baixo Alentejo region, Monte do Vento, Mértola, Southern Portugal (37º 48' 28.17" N/ -7º 40' 39.08" W), in a parcel of holm oak forest of *Quercus rotundifolia* L., where spontaneous *C. ladanifer* plants are the predominant vegetation (Supplementary material – *C. ladanifer* aerial parts). In the present work two groups of *C. ladanifer* aerial part samples were used: 1) those that emerged during current growth season (denominated as "young plants"); and 2) those with 2-6 growth seasons (plants with 2-6 years old, which were denominated as "old plants"). Samples of both groups were randomly collected during four consecutively seasons, with about 3 months between each sampling, i.e. spring (April), summer (July), autumn (October) and winter (January). The minimum and maximum air temperatures were: 12 and 24 ºC in April, 15 and 32 ºC in July, 15 and 27 ºC in October, and 5 and 15 ºC in January (data obtained at the nearest meteorological station, 37º45'20.5"N/08º04'35.4"W). Samples were manually harvested with scissors and pooled together into three samples (ca. 2 kg of plant material per pool). Immediately after collection, plant material was transported to laboratory and frozen at -20 ºC until processing. The plant material was then dried in ventilated over at 65 °C until constant weight, and ground using a mill with a sieve of 1 mm. The collected aerial parts were composed of leaves, soft stems, and reproductive organs (flower buds, flowers and fruits). The *C. ladanifer* shrubs showed leaves during the entire year, flower buds formation began at the end of winter, flowering occurred between March and April, and fruits were observed from May to November.

### 3.2.3 Fatty acid analysis

Fatty acid methyl esters of *C. ladanifer* ground samples (1 mm) were prepared by one-step transesterification procedure, according to Sukhija and Palmquist (1988) and modified by Palmquist and Jenkins (2003). Briefly, 1 mL of internal standard (19:0, 1 mg/mL in hexane) and 1 mL of toluene were added to 250 mg of sample, followed by the addition of 3 mL of 10% HCl solution in methanol (prepared by the addition of acetyl chloride to the methanol). After homogenization with a vortex mixer at slow speed, samples were maintained for 2 h at 70 ºC in a water bath. Thereafter, the solution was left to cool at room temperature and subsequently neutralized with 5 mL of 6% potassium carbonate. FAME were extracted with 2 mL of hexane and dried over 0.5 g of anhydrous sodium sulfate. Finally, samples were centrifuged for 5 min at 350 × g, the supernatant was transferred to new tubes and the solvent removed under nitrogen at 37ºC. The final residue was dissolved in 1.5 mL of hexane, and stored at -20 ºC until GC analysis. Additionally, a few samples (1-2 mg FAME) were hydrogenated with 1.5 mg of Adams catalyst (platinum dioxide) in 1mL of methanol under hydrogen at 50ºC for 1 hour, after cooling samples were filtrated to remove the catalyst and analyzed by GC.

### 3.2.4 Gas chromatography with flame ionization detection

FAME were analyzed by gas chromatography with flame ionization detection using a Shimadzu GC-2010 Plus chromatograph (Shimadzu, Kyoto, Japan) equipped with a 100% cyanopropyl polysiloxane capillary column (TR-CN100, 100 m, 0.25 mm i.d., 0.20 µm film thickness, Teknokroma, Barcelona, Spain). Helium was used as the carrier gas at a constant flow rate of 1 mL/min, and the injector and detector temperatures were 250 and 280 ºC, respectively. The split ratio was 1:50 and the injection volume was 1 µL. The column oven programmed temperature were as follow: initial oven temperature of 50 ºC was held for 1 min, increased to 150 ºC at 50 ºC/min and held for 20 min, then increased to 190 ºC at 1 ºC/min, and finally increased to 220 ºC at 2 ºC/min and maintained for 18 min.

### 3.2.5 Gas chromatography-mass spectrometry

Identification of FAME in *C. ladanifer* samples was achieved by electron impact mass spectrometry using a Shimadzu GC-MS QP2010 Plus (Shimadzu, Kyoto, Japan) equipped with a 100% cyanopropyl polysiloxane capillary column (CP-Sil88, 100 m, 0.25 mm i.d., 0.20 µm, film thickness, Agilent Tech., Wilmington, DE, USA). The gas chromatographic conditions were similar to the GC-FID conditions. The mass spectrometer conditions were as follows: ion

source temperature, 200 ºC; interface temperature, 220 ºC; ionization energy, 70 eV; scan, 50–500 atomic mass units. Unknown compounds in *C. ladanifer* samples were putatively identified by analysis of their mass spectrum and by using the NIST Mass Spectral library (2008).

### 3.2.6 Statistical analysis

Fatty acid composition was analyzed using the PROC MIXED option of SAS (SAS Institute Inc., Cary, NC, USA). Data were analyzed using a completely randomized design with four seasons (spring, summer, autumn and winter) 2x plant age groups (*C. ladanifer* plants from current growth season and *C. ladanifer* plants with 2-6 growth seasons) in a factorial arrangement with three repetitions (pooled samples). Least square means and standard error of mean (SEM) are presented in Table 3.1.

## 3. 3 Results

### 3.3.1 Fatty acid composition of *Cistus ladanifer*

The fatty acid composition of *C. ladanifer* aerial parts is dominated by saturated fatty acids (SFA). Indeed, 10 SFA (12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0 and 24:0) were identified in *C. ladanifer* samples, comprising more than 70% of total fatty acids. In contrast, only three unsaturated fatty acids were detected in *C. ladanifer* samples, i.e., the 18:1*cis*-9, 18:2n-6 and 18:3n-3. Surprisingly, two odd mono-methyl branched chain fatty acids (BCFA), the iso-19:0 and iso-21:0, were detected in the studied samples (Figure 3.1). The identity of both BCFA was confirmed by comparison with pure and spiked standards (Figure 3.1), and by mass spectrometry analysis (Figure 3.2). The mass spectrum of the iso-19:0 and iso-21:0 (Figure 3.2) presents the molecular ion at *m/z* 312 and 340, respectively, and an abundant $[M-43]^+$ ion corresponding to fragmentation to the side of the methyl branched (*m/z* 269 and *m/z* 297, respectively), as well as the ions at *m/z* 74, 87, 143, and 199, characteristic of SFA methyl esters (http://lipidlibrary.aocs.org). In addition, some samples were hydrogenated to eliminate unsaturated fatty acids that might co-elute with the BCFA, and then samples and standards were run in two different chromatographic columns (TR-CN100 and SLB-IL111) in order confirm the retention time of the iso-19:0 and iso-21:0. Since the occurrence of BCFA in plants is rather unusual, we tried to confirm whether their presence in our samples were not derived from any contamination from the soil or during sampling, or even if it was not a local peculiarity. So, we also analyzed *C. ladanifer* samples (collected by others) from another region of Portugal

(39º30'36"N/8º19'00"W). After analyzing these samples we detected again the presence of the BCFA (data not shown) in *C. ladanifer* samples.

Furthermore, as shown in Figure 3.1A, several unknown peaks were detected in *C. ladanifer* samples, which were later identified as labdane compounds. The labdane-type compounds are diterpenes with a similar bicyclic core skeleton (examples are presented in Figure 3.3), and they were named so, because they were first obtained from the labdanum, an oleoresin derived from *Cistus* plants. So, the mass spectra of the unknown peaks detected in our samples (Figure 3.3) present a similar molecular ion at *m/z* 320 and a fragment pattern typical of labdane-type compounds (Enzell & Ryhage, 1965). i.e., the fragment $[M-15]^+$ at *m/z* 305 corresponding to loss of a methyl group, the fragment $[M-31]^+$ at *m/z* 289 corresponding to loss of the methoxy group, and a series of ions formed by elimination of part of the first ring at *m/z* 223 ($[M-97]^+$), *m/z* 235 ($[M-85]^+$), *m/z* 249 ($[M-71]^+$), *m/z* 264 ($[M-56]^+$). In addition, peak 1 showed the base peak at *m/z* 191 formed by cleavage of the C9-C11 bond, as a result it was identified as the methyl labd-8-enoate. This compound has been previously characterized by Domenech-Carbo *et al*. (2009) and David *et al*. (2007). According to Enzell and Ryhage (1965), a characteristic fragmentation of labdanes having a C8-C17 double bond is the cleavage of the allylic C6-C7 and C9-C10 bonds accompanied by hydrogen transfer producing an intense ion at *m/z* 137, which was detected in mass spectrum of peak 2 (Figure 3.3). In addition, the prominent ion at *m/z* 177, corresponding to the loss of a methyl group from the fragment produced by cleavage of the C9-C11 bond, allowed the identification of peak 2 as the eperuic acid methyl ester. Finally, peak 3 was identified as the cativic acid methyl ester according to its mass spectrum (Figure 3.3), particular by the intense ions at *m/z* 196, 122 and 109, which were demonstrated by Enzell and Ryhage (1965) to be formed by fragmentation of retro-Diels Alder type for labdanes having a double bond at C7-C8.

**Figure 3.1.** Partial gas-liquid chromatogram of the fatty acid methyl esters separation using the TR-CN100 capillary column of 100-m long.
A Cistus ladanifer sample; B Cistus ladanifer sample spiked with iso-19:0 and iso-21:0 commercial standards; C Fatty acid methyl esters commercial standard mix.
The chromatographic conditions are presented in the "Materials and Methods"

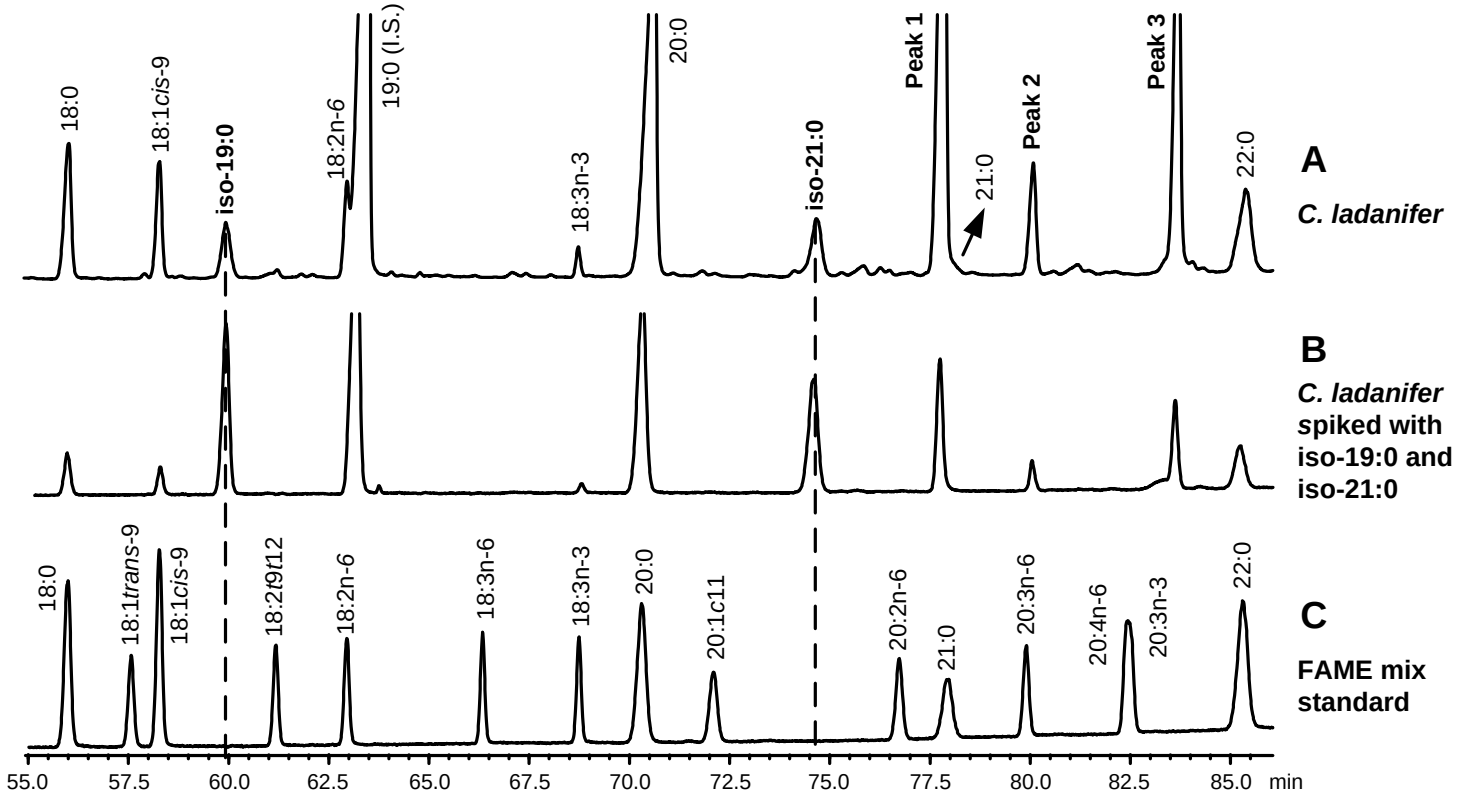


**Figure 3.2.** Electron impact mass spectra of fatty acid methyl esters derivatives of iso-19:0 and iso-21:0 detected in *Cistus ladanifer* samples.
The mass spectrometry conditions are presented in the "Materials and Methods"

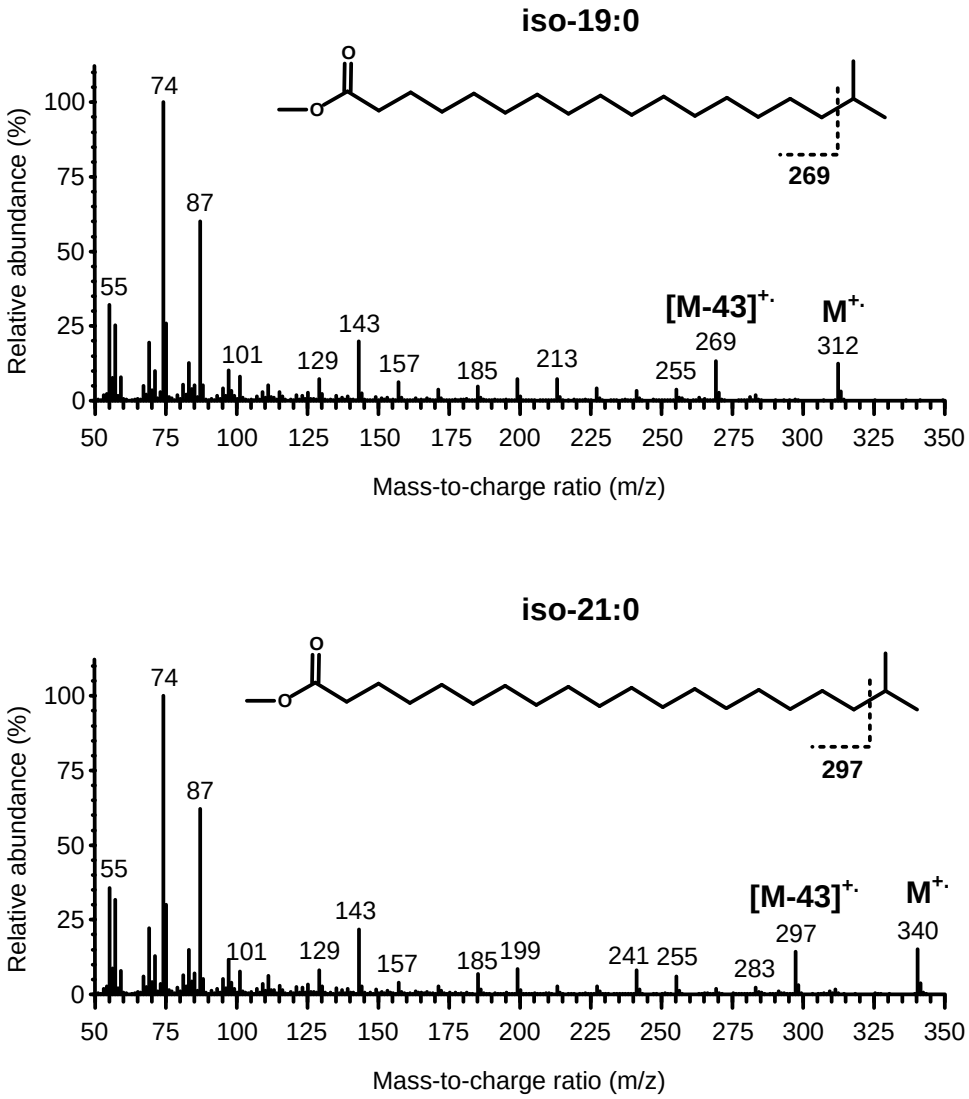
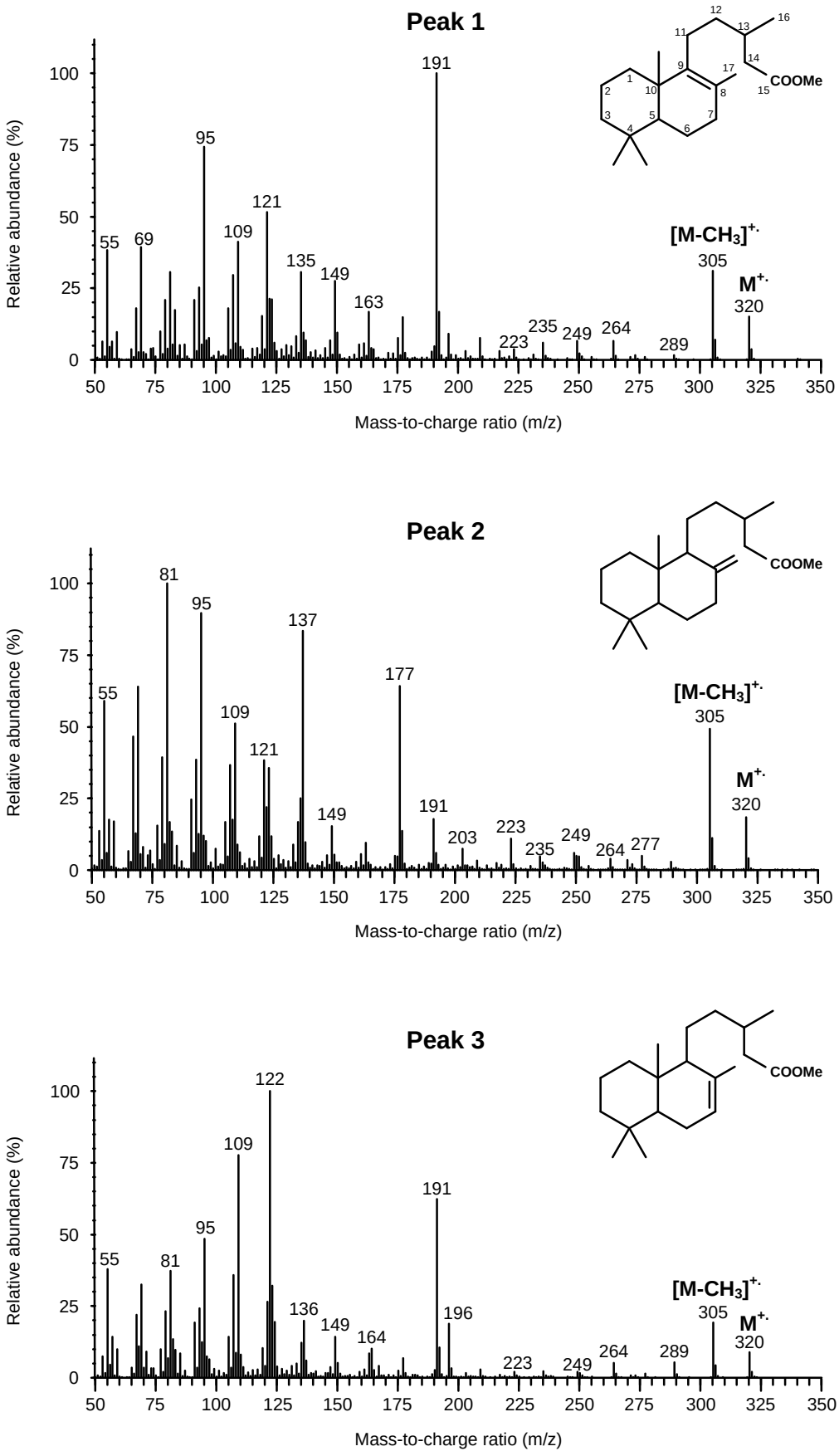


**Figure 3.3.** Electron impact mass spectra of labdane type-compounds in *Cistus ladanifer* samples. The mass spectrometry conditions are presented in the "Materials and Methods"



### 3.3.2 Effect of season and plant age

The fatty acid composition of *C. ladanifer* aerial parts on different seasons and plant ages is presented in Table 3.1. The total fatty acid content was affected ($P < 0.01$) by season, age and their interaction, being the highest content found in autumn, averaging 8.6 mg/g DM, and the lowest content was found in spring and winter, averaging 5.4 mg/g DM. In general, a low fatty acid content was found in *C. ladanifer*, which contrast with the fairly high ether extract content (ranging from about 50 to 90 mg/g DM) (Guerreiro *et al*., unpublished), indicating that fatty acids comprises only about 10% of the ether extract.

Interactions between season and plant age were found for most of fatty acids and the exceptions were the 15:0, 18:1*cis*-9 and 18:2n-6. In general a similar seasonal variation pattern is presented in both young and older plants, and interactions arise from more accentuated seasonal changes in young plants for most of the saturated fatty acids and 18:3n-3 than in older plants, whereas older plants showed larger seasonal changes in iso-19:0, and iso-21:0 than young plants.

The seasonal variation pattern is characterized by a large increase in summer and autumn of the 18:0, 20:0, 21:0, 22:0, iso-19:0 and iso-21:0, whereas PUFA have larger concentrations in winter and lowest in summer. For older plants, in autumn and also in summer, the total content of fatty acids increases from the basal levels of winter and spring. In average the increase in fatty acid concentration from spring to autumn is 3.3 mg/g DM, of which 2.0 mg/g (about 60%) was the 20:0, and 0.46 mg/g (about 15%) were BCFA, whereas the 16:0 displays a net decrease of -0.28 mg/g (about -8.6%) and polyunsaturated fatty acids (PUFA) display only modest increases (4% for 18:2n-6 and 3% for 18:3n-3).

**Table 3.1.** Effect of season and plant age on total fatty acid composition (mg/g DM) of *Cistus ladanifer* L. aerial parts

| | Spring | | Summer | | Autumn | | Winter | | SEM | *P* values | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Young | Old | Young | Old | Young | Old | Young | Old | | Season (S) | Age (A) | S×A |
| Total FA | 5.04$^{c}$ | 5.57$^{c}$ | 5.89$^{c}$ | 7.42$^{b}$ | 8.95$^{a}$ | 8.24$^{ab}$ | 5.26$^{c}$ | 5.53$^{c}$ | 0.190 | <0.001 | 0.008 | <0.001 |
| 12:0 | 0.02$^{cd}$ | 0.03$^{b}$ | 0.02$^{d}$ | 0.02$^{d}$ | 0.05$^{a}$ | 0.03$^{bcd}$ | 0.03$^{bc}$ | 0.02$^{cd}$ | 0.002 | <0.001 | <0.001 | <0.001 |
| 14:0 | 0.19$^{cd}$ | 0.23$^{b}$ | 0.17$^{d}$ | 0.20$^{cd}$ | 0.27$^{a}$ | 0.22$^{bc}$ | 0.13$^{e}$ | 0.12$^{e}$ | 0.007 | <0.001 | 0.94 | <0.001 |
| 15:0 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.001 | 0.001 | 0.32 | 0.07 |
| 16:0 | 1.34$^{b}$ | 1.48$^{a}$ | 1.01$^{e}$ | 1.24$^{bc}$ | 1.04$^{e}$ | 1.23$^{cd}$ | 1.18$^{cd}$ | 1.12$^{de}$ | 0.023 | <0.001 | <0.001 | <0.001 |
| 17:0 | 0.02$^{ab}$ | 0.03$^{ab}$ | 0.02$^{ab}$ | 0.03$^{a}$ | 0.02$^{ab}$ | 0.03$^{ab}$ | 0.02$^{ab}$ | 0.02$^{b}$ | 0.002 | 0.39 | 0.026 | 0.013 |
| 18:0 | 0.34$^{d}$ | 0.51$^{b}$ | 0.43$^{bc}$ | 0.63$^{a}$ | 0.72$^{a}$ | 0.62$^{a}$ | 0.36$^{cd}$ | 0.35$^{cd}$ | 0.020 | <0.001 | <0.001 | <0.001 |
| 18:1*cis*-9 | 0.21 | 0.29 | 0.44 | 0.53 | 0.15 | 0.24 | 0.09 | 0.15 | 0.036 | <0.001 | 0.008 | 0.98 |
| 18:2n-6 | 0.38 | 0.44 | 0.20 | 0.37 | 0.47 | 0.61 | 0.74 | 0.65 | 0.049 | <0.001 | 0.05 | 0.06 |
| 18:3n-3 | 0.29$^{b}$ | 0.23$^{bc}$ | 0.06$^{d}$ | 0.14$^{cd}$ | 0.37$^{ab}$ | 0.35$^{ab}$ | 0.44$^{a}$ | 0.29$^{b}$ | 0.029 | <0.001 | 0.07 | 0.007 |
| i-19:0 | 0.07$^{c}$ | 0.09$^{c}$ | 0.18$^{b}$ | 0.29$^{a}$ | 0.28$^{a}$ | 0.28$^{a}$ | 0.05$^{cd}$ | 0.04$^{d}$ | 0.008 | <0.001 | <0.001 | <0.001 |
| 20:0 | 1.33$^{f}$ | 1.48$^{ef}$ | 2.10$^{d}$ | 2.64$^{c}$ | 3.74$^{a}$ | 3.07$^{b}$ | 1.37$^{f}$ | 1.86$^{de}$ | 0.086 | <0.001 | 0.05 | <0.001 |
| i-21:0 | 0.16$^{c}$ | 0.13$^{cd}$ | 0.29$^{b}$ | 0.38$^{a}$ | 0.40$^{a}$ | 0.42$^{a}$ | 0.10$^{d}$ | 0.09$^{d}$ | 0.012 | <0.001 | 0.022 | <0.001 |
| 21:0 | 0.05$^{de}$ | 0.04$^{de}$ | 0.04$^{e}$ | 0.07$^{cd}$ | 0.13$^{a}$ | 0.11$^{ab}$ | 0.09$^{bc}$ | 0.09$^{bc}$ | 0.006 | <0.001 | 0.89 | 0.005 |
| 22:0 | 0.45$^{ef}$ | 0.38$^{f}$ | 0.62$^{bc}$ | 0.57$^{cd}$ | 0.86$^{a}$ | 0.68$^{b}$ | 0.45$^{ef}$ | 0.50$^{de}$ | 0.015 | <0.001 | <0.001 | <0.001 |
| 24:0 | 0.19$^{d}$ | 0.21$^{d}$ | 0.30$^{bc}$ | 0.31$^{bc}$ | 0.46$^{a}$ | 0.35$^{b}$ | 0.19$^{d}$ | 0.24$^{cd}$ | 0.015 | <0.001 | 0.48 | <0.001 |
| Partial sums | | | | | | | | | | | | |
| n-SFA | 3.93$^{d}$ | 4.39$^{cd}$ | 4.73$^{c}$ | 5.70$^{b}$ | 7.30$^{a}$ | 6.34$^{b}$ | 3.84$^{d}$ | 4.33$^{cd}$ | 0.145 | <0.001 | 0.030 | <0.001 |
| BCFA | 0.23$^{c}$ | 0.22$^{c}$ | 0.47$^{b}$ | 0.68$^{a}$ | 0.67$^{a}$ | 0.70$^{a}$ | 0.15$^{cd}$ | 0.12$^{d}$ | 0.019 | <0.001 | 0.002 | <0.001 |
| PUFA | 0.67$^{bc}$ | 0.67$^{bc}$ | 0.25$^{d}$ | 0.51$^{cd}$ | 0.83$^{abc}$ | 0.96$^{ab}$ | 1.18$^{a}$ | 0.93$^{ab}$ | 0.077 | <0.001 | 0.551 | 0.026 |

Young, plants that emerged during current growth season; Old, plants with between 2 and 6 growth season; FA, fatty acid; n-SFA, saturated fatty acids with linear chain; BCFA, branched chain fatty acids; PUFA, polyunsaturated fatty acids. SEM, standard error of the mean. Means with different letters within the same row are statistically different ($P < 0.05$).

## 3. 4 Discussion

The fatty acid composition of the *C. ladanifer* has been scarcely studied despite their use in animal nutrition studies to improve the nutritional value of meat fat due to the effect of *Cistus* tannins on the modulation of rumen biohydrogenation (Francisco *et al.*, 2015; Jerónimo *et al.*, 2012; Jerónimo *et al.*, 2010). As far as we know, only one study reported the fatty acid composition of *C. ladanifer* leaves (Guimarães *et al.*, 2009). In that study, the PUFA of *C. ladanifer*, collected in Northeastern Portugal, were reported to be more than 60% of total fatty acids, and 20:2n-6 was reported to be the major PUFA. However, it is known that plants rarely contain long chain PUFA because they do not have the enzymes necessary for their production (Guil-Guerrero, 2014), and such high levels of PUFA have only been found in transgenic plants modified to produce PUFA (Ruiz-Lopez, Sayanova, Napier, & Haslam, 2012). Also, as recently highlighted by Guil-Guerrero (2014), some misidentifications of PUFA on higher plants have been reported, particular when fatty acids are identified only by comparing the relative GC-FID retention time, of the supposed FAME peak, with the peaks from FAME standards. In our study, several unknown peaks were detected in the FAME chromatograms of *C. ladanifer* samples. Some of these peaks can be easily misidentified as 21:0 and 20:3n-6 fatty acids if only the relative retention time of FAME standards is used for peak identification (Figure 3.1C). However, the GC-MS analysis demonstrated that the unknown peaks were not FAME but diterpene compounds from the labdane type. The presence of labdane diterpenes in *C. ladanifer* leaves and essential oil have been already reported by several authors (Depascualt *et al.*, 1982; Gomes *et al.*, 2005; Martins *et al.*, 2014) and some labdanes have been shown to present antimicrobial, cytotoxic and antitumor activities (Papaefthimiou *et al.*, 2014). The methyl labdenoates detected in our samples have been identified by others after acidic methylation of plant extracts (David *et al.*, 2007; Depascualt *et al.*, 1982; Weyerstahl, Marschall, Weirauch, Thefeld, & Surburg, 1998). Therefore, because we also used acidic methylation for the preparation of FAME, these methyl labdenoates could have also been produced from labdenoic acids present in the plant. Nevertheless, we notice that they can interfere with the fatty acid analysis of plants rich in diterpene compounds.

Surprisingly, we detected the occurrence of BCFA in *C. ladanifer*, which are rarely found in the lipid fraction of higher plants. As far as we know, only two studies reported the occurrence of significant amounts of BCFA in plants, but none of these studies identified the iso-19:0 and iso-21:0. Tsydendambaev *et al.* (2004) identified eight iso-BCFA (iso-15:0, -16:0, -17:0, -18:0, -20:0, -22:0, -24:0, and -26:0) on the green above-ground parts of four alpine plants, and Radunz (1987) identified considerable quantities of iso-12:0, -14:0, -16:0, -18:0, and -22:0

BCFA in petals and chlorophyll-deficient leaves (averaging 9.0 and 4.2% of total FA, respectively) of *Antirrhinum majus* and *Nicotiana tabacum* plants. In addition, BCFA have been identified in epi-cuticular waxes of several plants (Arrendale, Severson, Chortyk, & Stephenson, 1988; Kroumova & Wagner, 1999).

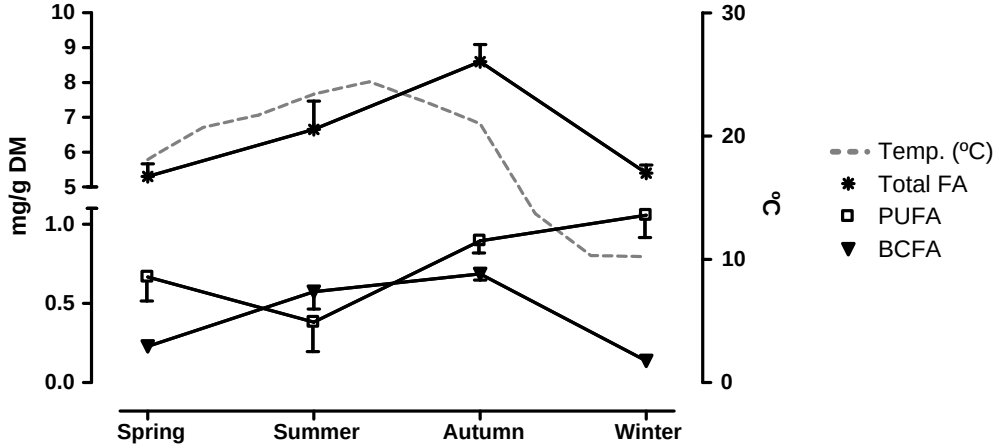
In ruminants, it is considered that odd and branched chain fatty acids in meat and milk fats are largely derived from bacteria within the rumen (Vlaeminck *et al.*, 2006). However, it seems that some plants may also contribute to the presence of BCFA in ruminant products, particularly iso-BCFA. Although the biosynthesis of odd-numbered iso fatty acids in higher plants are not completely known (Tsydendambaev *et al.*, 2004), they may be synthesized via C2-elongation of 3-methyl-butanoyl-(isovaleryl-)CoA derived from L-leucine via transamination and decarboxylation, similar to what was found to other organisms (Kaneda, 1977). Moreover, there is increasing interest in the BCFA as they showed antitumoral activity in human cancer cells (Wongtangtintharn, Oku, Iwasaki, & Toda, 2004) and also reduce the incidence of necrotizing enterocolitis, a devastating intestinal disease affecting premature infants (Ran-Ressler *et al.*, 2011). Therefore, the use plants rich in BCFA in the diet of ruminants may have positive healthy effects in ruminants and consequently on their products.

A clear opposite seasonal variation between the major SFA (i.e. 20:0 and 16:0) as well as for the BCFA and PUFA was observed in *C. ladanifer* at both ages. In winter and spring, *C. ladanifer* plants contain less fatty acids but more PUFA, as the hot Mediterranean summer proceeds, the fatty acids concentration increases mostly due to accumulation of 20:0 and BCFA and this trend peaks at middle of autumn, following the change of the environmental temperature in the sampling region (Figure 3.4). In a previous report where lambs were fed forage diets supplemented with three tannin sources, including *C. ladanifer*, it was observed that the highest content of 20:0 in neutral and polar meat lipids was detected in lambs fed diets with *C. ladanifer* (Jerónimo *et al.*, 2010). So, a relevant amount of 20:0 found in meat from those animals could be provided from the *C. ladanifer* shrub.

Moreover, our results are in agreement with other plants species (Falcone, Ogas, & Somerville, 2004; Pearcy, 1978), where the concentration of SFA increases and the PUFA (in particular the 18:3n-3) decreases as a response of plant adaptation to high growth temperatures. Indeed, plants have the ability to change the membrane fatty acid composition in response to temperature variations (Falcone *et al.*, 2004). In *C. ladanifer*, higher temperatures seem to promote the synthesis of BCFA and depletion of PUFA (Figure 3.4). Randunz (1987) suggested that BCFA can replace PUFA in the membrane phospholipids, and participate on regulation of plant membrane fluidity. We isolated the phospholipid fraction of only a few samples and detected

that the increase of BCFA in summer and autumn is associated with a clear increase of their incorporation in phospholipids and a decrease of PUFA (data not shown). So the replacement of PUFA by BCFA in *C. ladanifer* membrane phospholipids might be an adaptive response to the hot seasonal temperatures of Mediterranean climate.

**Figure 3.4** Variation in total fatty acid (FA), polyunsaturated fatty acid (PUFA) and branched chain fatty acid (BCFA) concentrations (mg/g DM) in *C. ladanifer* according to season and temperature (ºC).




The *C. ladanifer* shrub contains about 6.5 mg/g DM of total fatty acids and 0.8 mg/g DM of PUFA, which is much lower compared to the content commonly found in fresh forage (20.1 and 11.0 mg/g DM, respectively), and even lower than the content of low quality hay (8.8 and 2.5 mg/g DM, respectively) (Glasser, Doreau, Maxin, & Baumont, 2013), thus *C. ladanifer* is not a major dietary source of PUFA for ruminants. Therefore, the increased contents of *trans* fatty acids in lamb meat observed when *C. ladanifer* is incorporated in lambs diet (Francisco *et al.*, 2015) could not be attributed to the increased PUFA intake but most probably to modulation of rumen biohydrogenation by tannins (Jerónimo *et al.*, 2010).

## Acknowledgements

The authors want to thank Fundação para a Ciência e Tecnologia (FCT) through research grants to Olinda Guerreiro (SFRH/BD/84406/2012), Eliana Jerónimo (SFRH/BPD/74527/2010) and Susana P. Alaves (SFRH/BPD/76836/2011) and through the PTDC/CVT/120122/2010 project. We also thank the COMPETE/QREN program for their financial support of the ALENT-09-0140-FEDER000705 project, PRODER program for PRODER-020311015068 project, and to Associação de Defesa do Património de Mértola (ADPM) for kindly supplying the *Cistus ladanifer L*. samples

# CHAPTER 4

## EFFECTS OF EXTRACTS OBTAINED FROM *CISTUS LADANIFER* L. ON *IN VITRO* RUMEN BIOHYDROGENATION

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Adapted from *Animal Feed Science and Technology* (2016), 219, 304-3012

(Available online at http://dx.doi.org/10.1016/j.anifeedsci.2016.06.024)

## Abstract

*Cistus ladanifer* (rockrose) is a tanniferous and aromatic shrub abundant in marginal fields of Mediterranean countries that when fed to lambs has been associated with changes in rumen biohydrogenation that led to an increased concentration of vaccenic acid (*t*11-18:1) in abomasal digesta and meat. It is not clear what type of secondary compounds present in *C. ladanifer* might be responsible for its effects on rumen biohydrogenation. Thus, we tested the effects of essential oil (EO), dichloromethane extract (DE), total phenolics (TP), non-tannin phenols (NTP) and condensed tannins (CT) fractions on *in vitro* rumen biohydrogenation. Five *in vitro* batch incubations replicates were conducted in Hungate tubes containing 60 mg of feed substrate (Control), or 60 mg of feed substrate plus 6 mg of each *C. ladanifer* extract incubated for 6 h with 6 mL of buffered ruminal fluid. Volatile fatty acids (VFA) and long chain fatty acids (FA) were analyzed at 0 and 6 h and the biohydrogenation of *c*9-18:1, *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:3 and the yield of biohydrogenation products were computed. The production of total VFA was not affected by the *C. ladanifer* extracts and averaged 6.87 mmol/L. The disappearance of *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:3 was higher in CT than in Control and EO ($P < 0.05$). The gain in 18:0 and *t*11-18:1, relative to initial subtract availability, was higher in CT than in control and EO ($P < 0.05$). Both CT and DE increased ($P < 0.05$) the *c*9,*t*11-18:2 yield, relative to initial subtract availability, compared with Control and EO treatments. The 10-oxo-18:0 was the major oxo FA found and was highest for CT and lowest in EO and DE treatments. The results showed that CT was the most effective fraction of *C. ladanifer* regarding its effects on rumen biohydrogenation, inducing larger accumulation of *t*11-18:1, which was achieved not by inhibition of its last reductive biohydrogenation step (i.e. formation of 18:0) but by the promotion of the initial biohydrogenation steps.


**Keywords:** *Cistus ladanifer*, condensed tannin, plant secondary metabolites, rumen biohydrogenation, fatty acids, biohydrogenation intermediates

## 4. 1 Introduction

In ruminants, dietary unsaturated fatty acids (FA) are extensively isomerized and hydrogenated by rumen microbiota, which leads to the low content of polyunsaturated FA (PUFA), and variable but ubiquitous presence of trans FA in their tissues and products. Some of trans FA derived, directly or indirectly, from rumen biohydrogenation pathways like vaccenic acid (*t*11-18:1) and rumenic acid (*c*9,*t*11-18:2) have been reported to exert anticarcinogenic activities and other health promoting effects in several biological models (Gebauer *et al.*, 2011; Lim *et al.*, 2014). Strategies to protect dietary PUFA from the rumen biohydrogenation or modulate the relative abundance of selected biohydrogenation products have been under investigation for the last five decades (Bessa, Santos-Silva, Ribeiro, & Portugal, 2000; Cook, Scott, Ferguson, & McDonald, 1970).

Plant secondary metabolites have been proposed as potential modulators of ruminal biohydrogenation. Compounds such as saponins, terpenes and condensed tannins have been reported to be able to modify the pattern of biohydrogenation products (Vasta & Bessa, 2012). Although the effect of condensed tannins on ruminal biohydrogenation is still inconclusive, these plant secondary compounds seem to be promising on inhibiting the last reductive step of rumen biohydrogenation, where *trans*-18:1 isomers are converted into stearic acid (18:0) (Buccioni *et al.*, 2011; Carreño *et al.*, 2015; Khiaosa-Ard *et al.*, 2009; Vasta *et al.*, 2009a). This would promote the rumen outflow of *t*11-18:1 and consequently the content of *c*9,*t*11-18:2 deposited in tissues or transferred into milk due to the endogenous conversion of *t*11-18:1 into *c*9,*t*11-18:2 by the stearoyl-CoA desaturase (Bessa *et al.*, 2015; Shingfield & Wallace, 2014).

*Cistus ladanifer* (rockrose) is a shrub quite abundant in marginal fields of Mediterranean countries, being often used by grazing ruminants, particularly during the season of pasture scarcity. The inclusion of 250 g/kg of aerial parts of *C. ladanifer* into an oil-supplemented high-forage diet led to a large increase in *t*11-18:1 and lower 18:0 in abomasal digesta of lambs, and a 2.2-fold increase in deposition of *c*9,*t*11-18:2 in muscle compared to other vegetable oil-supplemented diets (Jerónimo *et al.*, 2010). More recently, we reported that the inclusion of *C. ladanifer* in a medium-forage diet increased the amount of *trans*-18:1 in lamb meat (Francisco *et al.*, 2015). We hypothesized that this effect was due to the very high amounts of condensed tannins in *C. ladanifer* (40 to 160 g/kg dry matter (DM) (Guerreiro *et al.*, 2016b)). Besides the condensed tannins, *C. ladanifer* also contains high levels of other types of secondary metabolites, including different phenolic and terpenoid compounds (Gomes *et al.*, 2005; Guerreiro *et al.*, 2015; Sosa *et al.*, 2005). Thus it is not clear which specific group of secondary compounds is responsible for the effects of *C. ladanifer* on rumen biohydrogenation. In order

to clarify which *C. ladanifer* group of secondary compounds is responsible for the changes in rumen biohydrogenation, we isolated several fractions from the whole plant (essential oils, dichloromethane and total phenolics, non-tannin phenols and condensed tannins extracts) and tested their effects on *in vitro* rumen biohydrogenation.

## 4. 2 Material and methods

### 4.2.1 *Cistus ladanifer* sampling

*Cistus ladanifer* aerial parts were harvested, in January 2013, in Baixo Alentejo region, in Monte do Vento, Mértola, Southern Portugal (37º 48' 28.17" N/ -7º 40' 39.08" W), in a parcel of holm oak forest of *Quercus rotundifolia* L., where spontaneous *C. ladanifer* plants are the predominant vegetation. Samples were manually harvested with scissors and kept at -20 ºC during 1 wk until further use. The collected aerial part of plants was composed of leaves and soft stems.

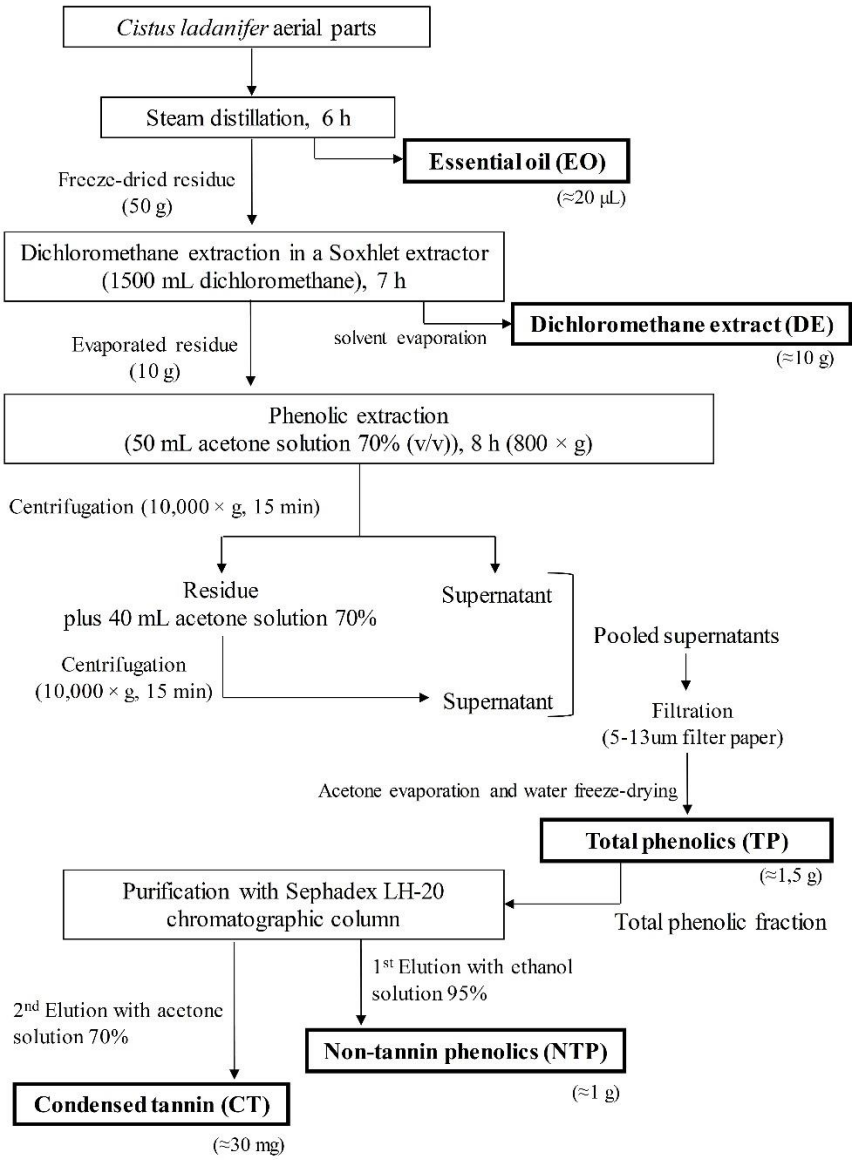
### 4.2.2 Preparation of secondary compounds fractions from *Cistus ladanifer*

Several fractions of secondary compounds were obtained by sequential extraction of *C. ladanifer* aerial parts (Figure 4.1). Samples (150 g) were submitted to steam distillation for 6 h, for extracting the essential oil (EO). However, in the present work a commercial *C. ladanifer* EO (*C. ladanifer* essential oil, Quinta Essência – Sociedade Agrícola Unipessoal, Lda, Évora, Portugal) was used, because the EO extraction yield was quite low. After steam distillation, the solid residue obtained was freeze-dried (FTS Systems, Inc., Stone Ridge, NY, USA) and ground using a mill with a sieve of 1 mm. Fifty grams of the solid residue was extracted with dichloromethane (1500 mL) in a Soxhlet extractor for 7 h. The dichloromethane extract (DE) was obtained by evaporating the solvent to dryness. Afterwards, 10 g of the leftover solid residue from dichloromethane extraction was further extracted with 50 mL of a mixture of acetone/water (70/30, v/v) at room temperature for 8 h, with gentle agitation (800g). The extract was then centrifuge at 10,000g for 15 min, and the supernatant collected. The leftover residue was further washed with 40 mL of the acetone/water mixture, and centrifuged at the same conditions describe above. Both supernatants were pooled, and then filtered through a 5-13 µm filter paper (VWR International, Carnaxide, Portugal). The fraction composed of all extracted phenolic compounds (total phenolics - TP) was obtained by sequential removal of acetone, by

rotary evaporation at 30ºC (VV2000, Heidolph instruments GmbH, Schwaback, Germany), and the water by freeze-drying.

The condensed tannins (CT) and non-tannin phenolics (NTP) were obtained from the TP fraction using a Sephadex LH-20 chromatographic column (GE Healthcare Bio-Science, Uppsala, Sweden), according to Strumeyer and Malin (1975) but with some modifications. Briefly, 200 mg of TP fraction, dissolved in 1 mL of a mixture of ethanol/water (80/20, v/v) was applied to a column of Sephadex LH-20 (2.3 × 27 cm) previously equilibrated with the ethanol/water mixture. The column was eluted with 400 mL of a mixture of ethanol/water (95/5, v/v) to collect the fraction of NTP. Then, the column was eluted with 200 mL of the acetone/water (70:30, v/v) mixture to collect the CT fraction. The solvents of both fractions (ethanol and acetone) were evaporated by rotator evaporation at 30ºC and the water was removed by freeze-drying.

**Figure 4.1.** Fractionation scheme followed for obtaining the *Cistus ladanifer* extracts



### 4.2.3 *In vitro* incubation with ruminal fluid

The rumen content from two rumen-fistulated Merino Branco rams fed daily 600 g of grass hay and 600 g of commercial concentrate, in two equal meals at 9:30 and 17:00 h. The concentrate mixture comprised maize, soybean, sunflower, wheat, wheat bran and rape (220 g crude protein; 95 g crude fibre; 65 g ash; 35 g ether extract; 4 g sodium; 7,500 IU of vitamin A; 1,500 IU of vitamin D3 and 7.5 mg of vitamin E; per kg of DM). Rumen fluid was collected just before the morning feeding in warm flasks (approximately 39 ºC) and strained through four layers of gauze. Strained ruminal fluid from both lambs was pooled and it was diluted with McDougall's buffer solution (McDougall, 1948), containing: 0.570 g/L of KCl, 0.470 g/L of NaCl, 0.120 g/L of MgSO4·7H2O, 0.040 g/L CaCl2, 9.80 g/L of NaHCO3 and 9.30 g/L Na2HPO4·12H2O; in a proportion of 1:2 (ruminal fluid:buffer solution, v/v), under constant $CO_2$ flux. After mixing, 6 mL of buffered ruminal fluid was added to Hungate tubes containing 60 mg of feed substrate (Control), or 60 mg DM of feed substrate plus 6 mg of each *C. ladanifer* fraction – EO, DE, TP, NTP and CT. The basal substrate used was a ground pelleted feed containing dehydrated alfalfa (700 g/kg), wheat grain (105 g/kg), soybean meal (110 g/kg) and sunflower oil (60 g/kg), and minerals and premix (25 g/kg). The chemical composition of the basal substrate was 902 g/kg DM, 175 g crude protein, 113 g starch, 81 g ether extract and 213 g crude fiber, per kg of DM. The final concentration of added *C. ladanifer* fractions was circa 100 g/kg DM. The tubes were then filled with $CO_2$, closed with a butyl rubber stoppers and screw cap. The DE and NTP fractions were previously dissolved in ethanol (60 mg/mL), being 100 µL of this solution added to the tubes containing substrate. The tubes allocated to all the other treatments also received 100 µL of ethanol, including the Control. Ethanol from all tubes was evaporated prior to incubation, overnight in an oven at 39 ºC. The tubes allocated to the 0 h incubation time were immediately frozen at -20 ºC after addition of the buffered ruminal fluid. Incubation was performed in a water-bath (Unitronic Pro, JP Selecta, Barcelona, Spain) at 39 ºC with gentle agitation, for 6h. At the end of incubation, tubes were immediately frozen at -20 ºC. For each treatment and incubation time, 2 Hungate tubes were used, one for volatile fatty acids (VFA) determination and other for long chain FA determination. The allocation of tubes to the treatments, incubation time, order of filling with buffered ruminal fluid, and to the position in the water-bath were randomized. The incubation procedure was replicated 5 times in 5 consecutive weeks.

Incubation tubes used for FA analysis were freeze-dried (ScanVac CoolSafe, LaboGene ApS, Lynge, Denmark), and stored at -20 ºC until analysis. Tubes for analysis of VFA were kept at -20 ºC until analysis.

### 4.2.4 Fatty acids analysis

Volatile FA (VFA) were analyzed by gas chromatography with flame ionization detection (GC-FID) using a Shimadzu GC-2010 Plus chromatograph (Shimadzu, Kyoto, Japan) equipped with a fused silica capillary column (Nukol, 30 m, 0.25 mm i.d., 0.25 µm film thickness, Sigma-Aldrich, St. Louis, MO), as described elsewhere (Oliveira, Alves, Santos-Silva, & Bessa, 2016).

The content of each incubation tube was transesterified to prepare FA methyl esters by using a combined basic followed by acid catalysis adapted from Jenkins (2010) and modified by Alves *et al*. (2013b), using as internal standard 19:0 (1 mg/mL). Fatty acid methyl esters were separated by GC-FID using a Shimadzu GC-2010 Plus chromatograph (Shimadzu, Kyoto, Japan) equipped with a cyanopropyl polysiloxane capillary column (TR-CN100, 100 m, 0.25 mm i.d., 0.20 µm film thickness, Teknokroma, Barcelona, Spain). Peak identification was based on comparison of retention times with FA methyl esters standards (37 component FAME mix and Bacterial acid methyl esters mix from Supelco, Sigma-Aldrich Inc., Bellefont, PA, USA) and by gas chromatography mass spectrometry using a Shimadzu GC-MS QP2010-Plus instrument (Shimadzu, Kyoto, Japan).

### 4.2.5 Calculations

In each incubation run and for each treatment a pair of tubes (i.e. one from 0 h and other from 6 h of incubation time) was available for VFA and C18 FA analysis. The VFA productions during the incubation time were calculated directly from the VFA concentration at 6 h minus 0 h incubation times.

Hereafter, the substrates of biohydrogenation metabolic pathways (BHS) are the unsaturated C18 FA derived from the diet (i.e. *c*9-18:1, *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:3) whereas the products of biohydrogenation (BHP) are the 18:0 and 18:1 and 18:2 isomers that present a proportional enrichment between 0 h and 6 h in fermenters. The disappearance of BHS, the formation of BHP, and their relative distribution were computed from their relative abundances at 0h and 6 h, expressed as g/kg of total C18 FA.

The disappearances of BHS, expressed as g/kg, were calculated as:

$$\text{Disappearance (g/kg)} = \frac{[S]_{0h} - [S]_{6h}}{[S]_{0h}} \times 1000$$

Where, $[S]_{xh}$ are the proportions at 0 h and 6 h of either *c*9-18:1, *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:3, expressed as g/kg of total C18 FA.

The yield (Y) of the major BHP normalized for initial availability of BHS, was calculated as proposed by (Jayanegara *et al.*, 2011):

$$Y_{18:0}\ (g/kg) = \frac{[18:0]_{6h} - [18:0]_{0h}}{[BHS]_{0h}} \times 1000$$

$$Y_{t11\text{-}18:1}\ (g/kg) = \frac{[\,t11\text{-}18:1]_{6h} - [t11\text{-}18:1]_{0h}}{[BHS]_{0h}} \times 1000$$

$$Y_{c9t11\text{-}18:2}\ (g/kg) = \frac{[c9t11\text{-}18:2]_{6h} - [c9t11\text{-}18:2]_{0h}}{[c9c12\text{-}18:2]_{0h}} \times 1000$$

Where, $[BHS]_{0h}$ is the sum of the proportion of *c*9-18:1, *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:3, present at 0 h, expressed as g/kg of total C18 FA.

The relative yield of each BHP ($RY_{BHP}$) expressed as g/kg of total BHP formed was calculated as:

$$RY\ (g/kg) = \frac{[P]_{6h} - [P]_{0h}}{[BHS]_{0h} - [BHS]_{6h}} \times 1000$$

Where, $[P]_{xh}$ are the proportions of each BHP at 0 or 6 h, expressed as g/kg of total C18 FA.

### 4.2.6 Statistical analysis

Data were analyzed using the Proc Mixed of SAS (SAS Institute inc, Cary, NC, USA), considering the fixed effect of treatments and the 5 incubations runs as random block. Least square means and standard error of the mean (SEM) are presented in tables. When significant treatment effects ($P < 0.05$) were detected multiple comparisons of means were conducted following Tukey's method.

## 4. 3 Results

The production of VFA (mmol/L) during incubation is presented in Table 4.1. In all treatments there was a similar net production of VFA, averaging 6.87 mmol/L across treatments. The production of individual VFA was also not influenced by *C. ladanifer* extracts, with exception of the acetate ($P = 0.016$), which increased in fermenter tubes with TP fraction compared with Control and EO treatments, and in NTP and CT compared with EO. The acetate:propionate ratio was increased ($P < 0.05$) by CT treatment compared with the other treatments except with TP.

**Table 4.1.** Effect of essential oil (EO), dichloromethane extract (DE), total phenolics (TP), non-tannin phenols (NTP) and condensed tannins (CT) of *Cistus ladanifer* on net production of volatile fatty acid (VFA, mmol/L) during the 6 h of incubation.

| | Control | EO | DE | TP | NTP | CT | SEM | *P* value |
|---|---|---|---|---|---|---|---|---|
| Total VFA | 6.4 | 6.0 | 7.3 | 7.6 | 7.2 | 6.7 | 0.52 | 0.169 |
| 2:0 | $3.0^{bc}$ | $2.7^{c}$ | $3.5^{abc}$ | $4.1^{a}$ | $3.6^{ab}$ | $3.8^{ab}$ | 0.31 | 0.016 |
| 3:0 | 1.8 | 1.8 | 2.2 | 2.1 | 2.1 | 1.7 | 0.22 | 0.097 |
| iso-4:0 | 0.20 | 0.15 | 0.17 | 0.15 | 0.17 | 0.10 | 0.056 | 0.851 |
| 4:0 | 1.06 | 1.00 | 1.11 | 0.95 | 0.98 | 0.90 | 0.147 | 0.479 |
| iso-5:0 | 0.17 | 0.15 | 0.16 | 0.11 | 0.14 | 0.08 | 0.038 | 0.140 |
| 5:0 | 0.19 | 0.20 | 0.20 | 0.16 | 0.15 | 0.18 | 0.029 | 0.586 |
| 2:0/3:0 ratio | $1.76^{bc}$ | $1.59^{c}$ | $1.65^{bc}$ | $2.05^{ab}$ | $1.82^{bc}$ | $2.29^{a}$ | 0.231 | 0.033 |

Means with different superscript letters within the same row are statistically different ($P < 0.05$).

The DE treatment led to a higher ($P < 0.05$) content of C18 FA compared with the other treatments, except for NTP at 0 h (Table 4.2). In 0 h fermenters these differences were due mostly to the *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:3 as the content of 18:0, *t*11-18:1, and *c*9-18:1 present in the 0 h fermenters tubes were similar ($P > 0.05$) among treatments. The FA and C18 FA contents (μg) in the fermenter tubes at 0 h did not differ from the 6 h tubes in all treatments ($P = 0.35$ for total FA and $P=0.97$ for the C18 FA, comparison not presented).

In the 6 h fermenter tubes, only 18:0 did not present differences ($P > 0.05$) among treatments (Table 4.2). The content of *c*9-18:1 and *c*9,*c*12-18:2 at 6 h was lower ($P < 0.05$) in CT than in DE, and the Control presented intermediate values. However, the content of *c*9,*c*12,*c*15-18:3 at 6 h was higher in DE compared with the other treatments. The EO presented lower ($P < 0.05$) *t*11-18:1 than CT, whereas the other treatment presented intermediate values. The Control and EO presented lower ($P < 0.05$) *c*9,*t*11-18:2 than DE, whereas the other treatment presented intermediate values.

The estimated biohydrogenation (disappearance) for dietary unsaturated C18 FA are presented in Table 4.3. The disappearance of *c*9-18:1 was consistently small in all treatments, although it was slightly but significantly larger ($P < 0.05$) in Control and CT than in EO and DE, and intermediate in TP and NTP treatments. The disappearance of *c*9,*c*12-18:2 (496 g/kg) was higher ($P < 0.05$) in CT than in Control (310 g/kg), EO (251 g/kg) and DE (347 g/kg), whereas TP and NTP presented intermediate values. Similarly, the disappearance of *c*9,*c*12,*c*15-18:3 (642 g/kg) was also higher ($P < 0.05$) in CT than in Control (453 g/kg) and EO (368g/kg), whereas DE and TP and NTP presented intermediate values.

**Table 4.2.** Effect of essential oil (EO), dichloromethane extract (DE), total phenolics (TP), non-tannin phenols (NTP) and condensed tannins (CT) of *Cistus ladanifer* on the concentration of C18 FA (µg per tube) at 0 and 6 h of incubation.

| | Control | EO | DE | TP | NTP | CT | SEM | *P* value |
|---|---|---|---|---|---|---|---|---|
| C18 FA, 0 h | | | | | | | | |
| 18:0 | 1164 | 1130 | 1167 | 1132 | 1176 | 1143 | 213 | 0.409 |
| *t*11-18:1 | 78 | 72 | 74 | 76 | 80 | 78 | 14.5 | 0.418 |
| *c*9-18:1 | 868 | 852 | 912 | 891 | 914 | 897 | 34.1 | 0.162 |
| *c*9,*c*12-18:2 | 1091$^{c}$ | 1216$^{bc}$ | 1470$^{a}$ | 1323$^{ab}$ | 1356$^{ab}$ | 1349$^{ab}$ | 76.1 | <0.001 |
| *c*9,*c*12,*c*15-18:3 | 120$^{d}$ | 138$^{cd}$ | 323$^{a}$ | 185$^{b}$ | 179$^{b}$ | 157$^{bc}$ | 11.1 | <0.001 |
| *c*9,*t*11-18:2 | 23$^{ab}$ | 22$^{b}$ | 21$^{b}$ | 25$^{ab}$ | 29$^{a}$ | 26$^{ab}$ | 2.7 | 0.012 |
| Total C18FA | 3467$^{c}$ | 3559$^{bc}$ | 4165$^{a}$ | 3756$^{bc}$ | 3855$^{ab}$ | 3767$^{bc}$ | 272 | <0.001 |
| C18 FA, 6h | | | | | | | | |
| 18:0 | 1451 | 1432 | 1514 | 1440 | 1423 | 1472 | 224 | 0.283 |
| *t*11-18:1 | 213$^{ab}$ | 185$^{b}$ | 260$^{ab}$ | 223$^{ab}$ | 257$^{ab}$ | 302$^{a}$ | 29.7 | 0.015 |
| *c*9-18:1 | 749$^{ab}$ | 789$^{ab}$ | 792$^{a}$ | 749$^{ab}$ | 760$^{ab}$ | 723$^{b}$ | 28.9 | 0.035 |
| *c*9,*c*12-18:2 | 785$^{ab}$ | 953$^{a}$ | 937$^{a}$ | 838$^{ab}$ | 851$^{ab}$ | 658$^{b}$ | 51.5 | 0.001 |
| *c*9,*c*12,*c*15-18:3 | 67$^{bc}$ | 91$^{b}$ | 161$^{a}$ | 87$^{b}$ | 83$^{bc}$ | 54$^{c}$ | 6.9 | <0.001 |
| *c*9,*t*11-18:2 | 26$^{b}$ | 28$^{b}$ | 67$^{a}$ | 40$^{ab}$ | 47$^{ab}$ | 52$^{ab}$ | 11.7 | 0.002 |
| Total C18FA | 3641$^{b}$ | 3756$^{b}$ | 4064$^{a}$ | 3699$^{b}$ | 3762$^{b}$ | 3636$^{b}$ | 251 | 0.004 |

Means with different superscript letters within the same row are statistically different ($P < 0.05$).

The yield of 18:0 and *t*11-18:1 per initial availability of biohydrogenation substrates ranged from 100 to 155 g/kg for 18:0 and from 46 to 99 g/kg for *t*11-18:1, being higher ($P < 0.05$) in CT than in Control and EO and intermediate in DE, TP and NTP (Table 4.3). The yield in *c*9,*t*11-18:2 per initial availability of *c*9,*c*12-18:2 was higher in DE (49 g/kg) and CT (41 g/kg) than in Control (5 g/kg) and EO (6 g/kg) and intermediate in TP and NTP.

The relative yield of BHP formed during the incubation is also presented in Table 4.3. Stearic acid (18:0) comprised 385 g/kg (Control) to 497 g/kg (DE) of total BHP formed and did not differ significantly among treatments, although in Control tended ($P = 0.06$) to be lower than in DE. Collectively, the 18:1 BHP comprised 393 g/kg (DE) to 479 g/kg (Control) of total BHP formed, with no significant differences among treatments. The treatment had no effect also on the relative contribution of *t*11-18:1 (which comprised about 230 g/kg of BHP formed) and of the majority of the other 18:1 isomers. Nevertheless, Control presented higher ($P < 0.05$) relative contribution of *c*12-18:1 than DE, and EO higher ($P < 0.05$) *t*9-18:1 than DE and higher ($P < 0.05$) *c*11-18:1 than NTP.

**Table 4.3.** Effect of essential oil (EO), dichloromethane extract (DE), total phenolics (TP), non-tannin phenols (NTP) and condensed tannins (CT) of *Cistus ladanifer* on disappearance of dietary unsaturated fatty acids (FA) and yield of biohydrogenation products (BHP)

| | Control | EO | DE | TP | NTP | CT | SEM | *P* value |
|---|---|---|---|---|---|---|---|---|
| Disappearance (g/kg) | | | | | | | | |
| *c*9-18:1 | 182$^{a}$ | 122$^{b}$ | 112$^{b}$ | 146$^{ab}$ | 151$^{ab}$ | 164$^{a}$ | 11.4 | <0.001 |
| *c*9,*c*12-18:2 | 310$^{b}$ | 251$^{b}$ | 347$^{b}$ | 356$^{ab}$ | 356$^{ab}$ | 496$^{a}$ | 33.9 | 0.001 |
| *c*9,*c*12,*c*15-18:3 | 453$^{b}$ | 368$^{b}$ | 487$^{ab}$ | 521$^{ab}$ | 518$^{ab}$ | 642$^{a}$ | 39.5 | 0.002 |
| BHP yields (g/kg)[1] | | | | | | | | |
| 18:0 | 106$^{b}$ | 100$^{d}$ | 144$^{ab}$ | 137$^{ab}$ | 119$^{ab}$ | 155$^{a}$ | 16.7 | 0.023 |
| *t*11-18:1 | 59.6$^{b}$ | 45.5$^{b}$ | 70.5$^{ab}$ | 61.7$^{ab}$ | 73.3$^{ab}$ | 98.6$^{a}$ | 13.07 | 0.009 |
| *c*9,*t*11-18:2 | 4.9$^{b}$ | 5.7$^{b}$ | 49.1$^{a}$ | 17.0$^{ab}$ | 25.4$^{ab}$ | 40.7$^{a}$ | 12.58 | 0.003 |
| BHP relative yields (g/kg)[2] | | | | | | | | |
| 18:0 | 385 | 458 | 497 | 466 | 438 | 407 | 52.0 | 0.060 |
| 18:1 isomers | | | | | | | | |
| *t*6-, *t*7-, *t*8- | 17.7 | 14.9 | 10.5 | 14.7 | 14.3 | 17.4 | 2.23 | 0.277 |
| *t*9- | 18.9$^{ab}$ | 23.9$^{a}$ | 9.4$^{b}$ | 12.9$^{ab}$ | 18.1$^{ab}$ | 17.6$^{ab}$ | 3.08 | 0.043 |
| *t*10- | 45.8 | 44.5 | 27.8 | 42.8 | 55.7 | 33.3 | 9.05 | 0.111 |
| *t*11- | 227 | 219 | 236 | 215 | 228 | 259 | 32.9 | 0.369 |
| *c*11- | 14.1$^{ab}$ | 21.8$^{a}$ | 16.2$^{ab}$ | 11.8$^{ab}$ | 9.2$^{b}$ | 12.5$^{ab}$ | 3.24 | 0.046 |
| *c*12- | 137$^{a}$ | 89$^{ab}$ | 73$^{b}$ | 81$^{b}$ | 90$^{ab}$ | 92$^{ab}$ | 19.0 | 0.026 |
| *t*16- | 9.8 | 12.0 | 9.2 | 9.8 | 9.5 | 9.2 | 1.34 | 0.431 |
| *c*15- | 6.0 | 7.1 | 8.9 | 9.0 | 11.1 | 7.5 | 1.22 | 0.106 |
| Sum | 479 | 435 | 393 | 399 | 438 | 451 | 44.9 | 0.098 |
| 18:2 isomers | | | | | | | | |
| *t*11,*c*15- | 9.0$^{ab}$ | 15.5$^{a}$ | 6.1$^{b}$ | 7.0$^{ab}$ | 7.5$^{ab}$ | 4.6$^{b}$ | 2.37 | 0.010 |
| *c*9,*t*11- | 7.5$^{b}$ | 10.9$^{b}$ | 55.4$^{a}$ | 23.2$^{b}$ | 22.5$^{b}$ | 32.6$^{ab}$ | 10.95 | <0.001 |
| *t*11,*c*13- | 4.9$^{a}$ | 6.1$^{a}$ | 0$^{b}$ | 7.3$^{a}$ | 9.0$^{a}$ | 7.2$^{a}$ | 1.25 | <0.001 |
| *t*11,*t*13- | 10.6$^{a}$ | 0$^{b}$ | 0$^{b}$ | 11.5$^{a}$ | 11.0$^{a}$ | 8.9$^{a}$ | 3.15 | <0.001 |
| *t*9,*t*11- | 18.0 | 12.2 | 10.6 | 17.4 | 17.9 | 21.2 | 3.87 | 0.088 |
| Sum | 50.0$^{ab}$ | 44.7$^{b}$ | 72.0$^{ab}$ | 66.3$^{ab}$ | 67.7$^{ab}$ | 74.5$^{a}$ | 12.42 | 0.024 |
| Oxo FA | | | | | | | | |
| 10-oxo-18:0 | 64.9$^{a}$ | 45.6$^{b}$ | 28.1$^{c}$ | 48.6$^{b}$ | 42.8$^{b}$ | 52.4$^{ab}$ | 4.96 | <0.001 |
| 13-oxo-18:0 | 21.3$^{a}$ | 17.4$^{ab}$ | 9.7$^{b}$ | 20.8$^{a}$ | 12.1$^{ab}$ | 14.2$^{ab}$ | 3.76 | 0.008 |
| Sum | 86.1$^{a}$ | 63.0$^{b}$ | 37.8$^{c}$ | 69.4$^{ab}$ | 55.0$^{bc}$ | 66.7$^{ab}$ | 7.88 | <0.001 |

Means with different superscript letters within the same row are statistically different ($P < 0.05$).
[1] BHP gain per initial substrate availability.
[2] Individual BHP gain as g/kg of total BHP gains.

Collectively, the 18:2 BHP comprised from 45 g/kg (in EO) to 75 g/kg (in CT) of total BHP. The proportion of 18:2 BHP products was larger in CT when compared to EO, and intermediate in the other treatments. The major individual 18:2 BHP isomer was the *c*9,*t*11-18:2 in DE, TP, NTP and CT treatments and the *t*9,*t*11-18:2 in Control and *t*11,*c*15-18:2 in EO. DE presented higher ($P < 0.05$) *c*9,*t*11-18:2 than the other treatments except CT that was intermediate.

Collectively, oxo-C18 FA comprised from 38 g/kg (in DE) to 86 g/kg (in Control) of the BHP formed. The major oxo-C18 FA was the 10-oxo-18:0 which was lower ($P < 0.05$) in DE than the other treatments, whereas Control presented the highest value, being higher ($P < 0.05$) than EO, TP and NTP but not different ($P > 0.05$) from CT. The 13-oxo-18:0 was higher in Control and TP ($P < 0.05$) than DE, whereas the other treatment presented intermediate values.

## 4. 4 Discussion

A small scale and short term *in vitro* batch incubation with strained rumen contents was used to test the effects of several *C. ladanifer* extracts on rumen biohydrogenation of soybean oil incorporated in the substrate. Rumen lipolysis and biohydrogenation kinetic studies reveal that these pathways can be quite fast (i.e. fractional rates ranging from 0.06/h to 0.40/h) (Beam, Jenkins, Moate, Kohn, & Palmquist, 2000; Enjalbert *et al.*, 2003; Jouany, Lassalas, Doreau, & Glasser, 2007) and that stable endpoints are reached as time proceeds. Thus, with long incubation times a similar biohydrogenation endpoints might be achieved regardless any treatments effects at earlier stages (Enjalbert *et al.*, 2003). However, short incubation times might not allow for a full adaptation of rumen microbial inoculum to the experimental conditions and eventually not capture the stable endpoints of end product (i.e. 18:0) accumulation. Thus, the short duration of the incubation must be taken in consideration when interpreting the results. The disappearance of *c*9,*c*12-18:2 observed was similar to that reported by Troegeler-Meynadier *et al*. (2014) using a 6 h incubation time and also similar to the disappearance predicted for 6 h using the disappearance rates (0.06-0.12/h) reported by Beam *et al*. (2000). Nevertheless, the disappearance of *c*9,*c*12-18:2 was comparatively lower than that predicted from *in vitro* kinetics data for 6 h reported by Enjalbert *et al*. (2003), Jouany *et al*. (2007) and Honkanen *et al*. (2012).

The amount of *C. ladanifer* extracts used in the incubation did not inhibit the general fermentative activity of rumen microorganism, as evaluated by the production of total VFA. The production of VFA was 1.15 mM/h which is comparable with what was reported in other *in vitro* batch incubation experiments (Castagnino *et al.*, 2015; Diaz, Ranilla, Giraldo, Tejido,

& Carro, 2015; Vasta *et al.*, 2009a). This is an important validation step, as very high doses of plant secondary compounds might inhibit the general microbial metabolism and thus also the biohydrogenation activity.

The present study suggests that CT was the most active fraction of *C. ladanifer* regarding its effects on rumen biohydrogenation, although similar but milder activity was observed for TP (which contains the CT) and NTP fractions. In fact, CT induced the largest increased on the disappearance of *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:3 and consequently the largest accumulation of *t*11-18:1 and 18:0. The biohydrogenation of *c*9,*c*12-18:2, the main substrate present in the fermenters, involves an initial isomerization, which produces *c*9,*t*11-18:2, and two sequential reductive steps, producing *t*11-18:1 and 18:0, respectively (Shingfield & Wallace, 2014). Condensed tannins have been reported to inhibit the last reductive step, and thus promoting the accumulation of *t*11-18:1 in detriment of 18:0 (Khiaosa-Ard *et al.*, 2009). Notably, no inhibition of the last reductive biohydrogenation step was observed for CT, as the relative yield of *t*11-18:1 and 18:0 remained similar among treatments. Thus, the increase in *t*11-18:1 induced by CT results from the increased activity of the initial steps of the biohydrogenation. The large variability of the effects of tannins on biohydrogenation could be explained by the chemical structural diversity of the tannins derived from different sources. However, the present results also contradicted our *in vivo* data, where lambs fed oil and *C. ladanifer* plant also presented in abomasal digesta an increase *t*11-18:1 associated with a decrease of 18:0 (Jerónimo *et al.*, 2010). Such differences may be related to the dose of tannins used, which can also modify its effects on ruminal biohydrogenation pattern as recently discussed by Carreño *et al*. (2015). These authors reported significant interactions between tannin source and dose (20 g/kg to 80 g/kg) on *in vitro* biohydrogenation. The lower doses of oak tannins seemed to inhibit the initial steps of biohydrogenation but this effect become less clear when oak tannins concentration increased (Carreño *et al.*, 2015). This pattern might also be applicable to *C. ladanifer* CT, as the concentration of CT in lambs diets containing *C. ladanifer* in the experiment reported by Jerónimo *et al*. (2010) was only 21 g/kg DM. In the present *in vitro* experiment we used a much higher tannin concentration (100 g/kg DM), resulting on an accumulation of *t*11-81:1 coupled with stimulation of the initial steps of biohydrogenation and no indication of the expected inhibition of the last reductive biohydrogenation step. Factors like the presentation of condensed tannins (purified *vs*. constitutive of vegetal tissues), interactions with the basal diet and features of *in vitro* procedures might explain the differential response patterns. For instance, the short duration of the incubation might explain the lack of effects on the formation of 18:0, as data from Buccioni *et al*. (2011) indicate that the tiny reduction of 18:0 concentrations on

solid associated rumen bacteria induced by tannins observed at 6 h of incubation was more clear as the incubation time is extended to 12 h and particularly to 18 h.

Besides CT, other plant secondary metabolites, such as terpenoids, which are present in EO, might also modulate the rumen biohydrogenation. Indeed, Gunal *et al*. (2013) reported an accumulation of *trans*-18:1 and a decrease of 18:0 due to inclusion of essential oils extracted from several plants on *in vitro* batch incubations with rumen fluid. Nevertheless, the *C. ladanifer* EO did not influence the ruminal biohydrogenation and its effect was restricted to only a reduction of 10-oxo-18:0 and *c*9-18:1 biohydrogenation.

The DE extract included the *C. ladanifer* lipids, but also terpenoids, pigments and many other uncharacterized compounds. In fact, *C. ladanifer* contains only low FA content (5.4-8.6 g/kg DM) but a high ether extract content (50- 90 g/kg DM) (Guerreiro *et al.*, 2015; Guerreiro *et al.*, 2016b). In spite of the fact that *C. ladanifer* lipids are particularly enriched with saturated FA (Guerreiro *et al.*, 2015), the inclusion of DE significantly increased the content of C18 unsaturated FA in fermenters. Increasing initial unsaturated FA concentration on *in vitro* batch systems inhibits the conversion of *trans*-18:1 into 18:0 (Harfoot, Noble, & Moore, 1973). Notably, DE induced the highest yield of *c*9,*t*11-18:2, which was coupled with the absence of formation of other conjugated isomers. Moreover, DE tended to present low relative yields of other BHP like *t*9-18:1, *c*12-18:1, *t*11,*c*15-18:2 and oxo-FA. This suggest that biohydrogenation pathways were less diversified favoring of *c*9,*t*11-18:2 and *t*11-18:1 in detriment of other BHP.

Two oxo-FA were identified as major BHP. There are a few reports of rumen *in vitro* studies that present the production of oxo-FA, and our results showed a higher proportion of oxo-FA than the reported by Jenkins *et al*. (2006) and Carreño *et al*. (2015). The 10-oxo- and 13-oxo-18:0 result probably from the metabolism of *c*9-18:1 and *c*9,*c*12-18:2, respectively. Several bacteria in the rumen are responsible for the hydration of *c*9-18:1 and further oxidation of 10-OH-18:0 (10-hydroxystearic acid) to 10-oxo-18:0 (Hudson, MacKenzie, & Joblin, 1995; Jenkins *et al.*, 2006; McKain, Shingfield, & Wallace, 2010). The association of reduced *c*9-18:1 biohydrogenation (i.e. disappearance) and reduced yield in 10-oxo-18:0 found for EO and DE treatments reinforce the plausibility that 10-oxo-18:0 is an end product of *c*9-18:1 ruminal metabolization. Rumen bacteria are also capable of converting *c*9,*c*12-18:2 to *c*9,13-OH-18:1, followed by reduction to 13-OH-18:0 (13-hydroxysteraic acid) and finally by oxidation to 13-oxo-18:0 (Alves *et al.*, 2013b; Kairenius, Toivonen, & Shingfield, 2011).

## 4. 5 Conclusion

The present work indicates that among the extracts obtained from *C. ladanifer* the CT is the most active in modulating the rumen biohydrogenation *in vitro*. At high dose and at short incubation times used, the effect of CT on increasing *t*11-18:1 yield was achieved not by inhibition of its last reductive biohydrogenation step (i.e. formation of 18:0) but by the promotion of the initial biohydrogenation steps. The DE fraction increased the yield and relative yield of *c*9,*t*11-18:2, probably by reducing the activity of biohydrogenation pathways that generate other BHP.

The present work reinforces the hypothesis that the modulatory effects of *C. ladanifer* plant on rumen biohydrogenation observed *in vivo* (Francisco *et al.*, 2015; Jerónimo *et al.*, 2010) are due to its high CT content.

## Acknowledgements

The authors want to thank Fundação para a Ciência e Tecnologia (FCT) through research grants to Olinda Guerreiro (SFRH/BD/84406/2012), Mónica Costa (SFRH/BD/90468/2012) and Susana P. Alves (SFRH/BPD/76836/2011) and through the PTDC/CVT/120122/2010 project.

# CHAPTER 5

## EFFECTS OF INCREASING DOSES OF CONDENSED TANNINS EXTRACT FROM *CISTUS LADANIFER* L. ON *IN VITRO* RUMINAL FERMENTATION AND BIOHYDROGENATION

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Article in advanced preparation to be submitted to *Animal Feed Science and Technology*

## Abstract

*Cistus ladanifer* (rockrose) is a perennial shrub quite abundant in Mediterranean region, a rich source in secondary compounds as condensed tannins (CT). Condensed tannins from *Cistus ladanifer* were able to change the ruminal biohydrogenation (BH), increasing the *t*11-18:1 and *c*9,*t*11-18:2 production. However, the levels of *C. ladanifer* CT that allows optimizing the production of *t*11-18:1 and *c*9,*t*11-18:2 is not yet known. Thus, we tested the effect of increasing doses of *C. ladanifer* CT extract (25 to 100 g/kg DM) on *in vitro* rumen BH. Five *in vitro* batch incubations replicates were conducted in Hungate tubes containing 60 mg of feed substrate (Control), or 60 mg DM of feed substrate plus each dose of *C. ladanifer* CT extract incubated for 24 h with 6 mL of buffered ruminal fluid. Volatile fatty acids (VFA) and long chain fatty acids (FA) were analyzed at 0 h and 24 h, and BH of *c*9-18:1, *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:3, and BH products yield were computed. Increasing levels of *C. ladanifer* CT extract reduced the total and individual VFA production with minor effect on BH. The BH of *c*9 -18:1 and *c*9,*c*12-18:3 was not affected, *c*9,*c*12,*c*15-18:3 BH suffered a minimal decrease, and the production of *t*11-18:1 and *c*9,*t*11:18:2 remained unchanged. Moreover, increasing levels of *C. ladanifer* CT extract induced to depression on OBCFA and DMA production, which was more accentuated than the reduction in the VFA production. Such result associated to maintenance of the BH, suggest that bacterial activities were differentially affected by *C. ladanifer* CT extract, with more pronounced effect on growth activities rather than on other microbial activities. The results observed in present work may be an adaptive response of microbial population to stress stimuli caused by high levels of CT.

**Keywords:** *Cistus ladanifer*, condensed tannin, rumen fermentation, fatty acids, biohydrogenation intermediates

## 5. 1 Introduction

Tannins are a group of plant secondary compounds, defined as naturally occurring water-soluble polyphenols that have ability to precipitate proteins (Bhat, Singh, & Sharma, 1998; Patra & Saxena, 2011). Tannins are generally classified as hydrolysable and condensed tannins (CT). Condensed tannin polymers vary tremendously in their constituent monomers, stereochemistry, polymer size, and intermolecular linkages, in addition to their concentration (McMahon *et al.*, 2000), and are considered as antinutritional factors due to their adverse effects on feed intake, nutrient utilization and toxicity (McSweeney *et al.*, 2001). However, CT have also been recognized as useful phytochemicals for modulating rumen microbial fermentation (Patra & Saxena, 2011), and ruminal biohydrogenation (BH) of dietary unsaturated fatty acids (FA) (Guerreiro *et al.*, 2016a; Khiaosa-Ard *et al.*, 2009; Vasta *et al.*, 2009a).

Ruminants edible products have been associated with adverse health effects, due to their high content of saturated FA (SFA) and low levels of polyunsaturated FA (PUFA) due to the extensive BH conducted by rumen microbiota. Therefore, manipulation of the ruminal BH has been attempted as way to improve the ruminant fat healthiness, by increase the rumen outflow of the dietary PUFA and the beneficial biohydrogenation intermediates, such as vaccenic acid (*t*11-18:1) and rumenic acid (*c*9,*t*11-18:2) (Bessa *et al.*, 2000; Vasta & Bessa, 2012). Some *in vitro* studies have suggested that CT may be efficient to inhibit the last step of BH, decreasing the 18:0 formation and leading to *t*11-18:1 accumulation (Buccioni *et al.*, 2011; Khiaosa-Ard *et al.*, 2009; Vasta *et al.*, 2009a). However, other studies reported a general inhibition of BH without the inhibition of it last step (Carreño *et al.*, 2015; Costa *et al.*, 2017a; Guerreiro *et al.*, 2016a).

*Cistus ladanifer* is a shrub widely spread over the Mediterranean region and often used by grazing ruminants, particularly during periods of pasture scarcity (Guerreiro *et al.*, 2016b). The inclusion of *C. ladanifer* into oil-supplemented diets modified the lamb abomasal and meat FA composition (Francisco *et al.*, 2015; Jerónimo *et al.*, 2012; Jerónimo *et al.*, 2010). These modulatory effects on BH should be associated with CT, due to its high amounts of CT (40-160 g/kg dry matter (DM) (Guerreiro *et al.*, 2016b) and by the fact that CT extract was the secondary compounds fraction of *C. ladanifer* that presented the highest capacity to modulate the BH pattern (Guerreiro *et al.*, 2016a). However, the effects of CT on BH might change with the dose as demonstrated by Buccioni *et al.* (2011) and Carreño *et al.* (2015). Thus, the present *in vitro* experiment was designed to determine the dose of *C. ladanifer* CT which optimize the *c*9,*t*11-18:2 and *t*11-18:1 production.

## 5. 2 Material and methods

### 5.2.1 *Cistus ladanifer* sampling

*Cistus ladanifer* aerial parts were harvested, in January 2013, in Baixo Alentejo region, in Monte do Vento, Mértola, Southern Portugal (37º 48' 28.17" N/ -7º 40' 39.08" W), in a parcel of holm oak forest of *Quercus rotundifolia* L., where spontaneous *C. ladanifer* plants are the predominant vegetation. Samples were manually harvested with scissors and kept at -20ºC during 1 wk until further use. The collected aerial part of plants was composed of leaves and soft stems.

### 5.2.2 Preparation of *Cistus ladanifer* condensed tannins extract

The condensed tannins (CT) extract were obtained by sequential extraction of *C. ladanifer* aerial parts and purified using a Sephadex LH-20 chromatographic column (GE Healthcare Bio-Science, Uppsala, Sweden), as described elsewhere (Guerreiro *et al.*, 2016a).

### 5.2.3 *In vitro* incubation with ruminal fluid

The rumen content from two rumen-fistulated Merino Branco rams fed daily 600 g of commercial concentrate and 600 g of grass hay, in two equal meals at 9:30 and 17:00 h. The concentrate mixture comprised maize, soybean, sunflower, wheat, wheat bran and rape (220 g crude protein; 95 g crude fibre; 65 g ash; 35 g ether extract; 4 g sodium; 7,500 IU of vitamin A; 1,500 IU of vitamin $D_3$ and 7.5 mg of vitamin E; per kg of DM). Rumen fluid was collected just before the morning feeding in warm flasks (approximately 39 ºC) and strained through four layers of gauze. Strained ruminal fluid from both lambs was pooled and it was diluted with phosphate-bicarbonate buffer solution (Goering & Van Soest, 1970), pre-warmed at 39 ºC and saturated with $CO_2$, in a proportion of 1:4 (ruminal fluid:buffer solution, v/v), under constant $CO_2$ flux. Before adding the ruminal fluid, a redox indicator (resazurin solution, 0.1% (w/v)) and a reducing agent (625 mg of L-cysteine-HCl, 1 N sodium hydroxide and 625 mg of sodium sulfide nonahydrate) were added to the buffer solution. Reduction was indicated upon change in color of the blue resazurin to colorless dihydroresorufin. After complete reduction of buffer solution and mixture with strained ruminal fluid, 6 mL of buffered ruminal fluid was added to Hungate tubes containing 60 mg of ground feed substrate (control), or 60 mg DM of feed substrate plus each dose of *C. ladanifer* CT extract (1.5, 3.0, 4.5 and 6 mg). The basal substrate was a mixture of commercial concentrate (564 g/kg), grass hay (376 g/kg) and sunflower oil

(60 g/kg). The commercial concentrate and grass hay were the same used to feed the rumen content donors. The final concentrations of added *C. ladanifer* CT extract were 25, 50, 75 and 100 g/kg DM, corresponding to 1.5, 3, 4.5 and 6 % of CT, respectively. The tubes were then filled with $CO_2$, thus ensuring oxygen-free conditions and closed with a butyl rubber stoppers and screw cap. The tubes allocated to the 0 h incubation time were immediately frozen at -20 ºC after addition of the buffered ruminal fluid. Incubation was performed in a water-bath (Unitronic Pro, JP Selecta, Barcelona, Spain) at 39 ºC with gentle agitation, for 24 h. At the end of incubation, tubes were immediately frozen at -20 ºC. For each treatment and incubation time, 2 Hungate tubes were used, one for volatile fatty acids (VFA) determination and other for long chain FA determination. The allocation of tubes to the treatments, incubation time, order of filling with buffered ruminal fluid, and to the position in the water-bath were randomized. The incubation procedure was replicated 5 times in 5 consecutive weeks.

Incubation tubes used for FA analysis were freeze-dried (ScanVac CoolSafe, LaboGene ApS, Lynge, Denmark), and stored at -20ºC until analysis. Tubes for analysis of VFA were kept at -20 ºC until analysis.

### 5.2.4 Fatty acids analysis

Volatile FA were analyzed by gas chromatography with flame ionization detection (GC-FID) using a Shimadzu GC-2010 Plus chromatograph (Shimadzu, Kyoto, Japan) equipped with a fused silica capillary column (Nukol, 30 m, 0.25 mm i.d., 0.25 µm film thickness, Sigma-Aldrich, St. Louis, MO), as described elsewhere (Oliveira *et al.*, 2016).

The content of each incubation tube was transesterified to prepare FA methyl esters by using a combined basic followed by acid catalysis adapted from Jenkins (2010) and modified by Alves *et al*. (2013b), using as internal standard 19:0 (1 mg/ml). Fatty acid methyl esters were separated by GC-FID using a Shimadzu GC-2010 Plus chromatograph (Shimadzu, Kyoto, Japan) equipped with a 100% cyanopropyl polysiloxane capillary column (SP-2560, 100 m, 0.25 mm i.d., 0.20 µm film thickness, Teknokroma, Barcelona, Spain). Peak identification was based on comparison of retention times with FA methyl esters standards (37 component FAME mix and Bacterial acid methyl esters mix from Supelco, Sigma-Aldrich Inc., Bellefont, PA, USA) and by gas chromatography mass spectrometry using a Shimadzu GC-MS QP2010-Plus instrument (Shimadzu, Kyoto, Japan).

### 5.2.5 Calculations

In each incubation run and for each treatment a pair of tubes (i.e. one from 0 h and other from 24 h of incubation time) was available for VFA and FA analysis. Data from each 0 h/24 h pair were used for computing the VFA and FA balance that were used in the statistical analysis. The VFA and FA (except C18 FA) productions during the incubation time were calculated directly from their concentrations at 24 h minus 0 h incubation times. The direct balance between the amounts present at 0 h and 24 h incubation times pairs of tubes in each treatment and incubation run, was not possible due to the random variation of the total amount of C18 FA estimated by the internal standard method in each independent tube of the 0 h and 24 h pairs. Thus, assuming that no C18 FA undergo through carbon chain elongation or shortening and that the *de novo* synthesis of C18 FA would be negligible, due to abundance of C18 FA in substrate (Demeyer, Henderson, & Prins, 1978) we averaged the content of C18 FA (in µg *per* tube) present in the pair of tubes (0 h and 24 h) of each treatment in each incubation run. The differences between 24 h and 0 h were then computed using the mean content of total amount of C18 FA in each pair of tubes and the relative distribution of C18 FA (in % of total C18 FA) present at the tube from 0 h and 24 h incubation time. The calculations for the balance of 18:0 are given here as example:

1) $[\sum C18_{m}] = ([\sum C18_{0h}] + [\sum C18_{24h}])/2$

2) $[18:0m_{0h}] = ([\sum C18_{m}] \times P(18:0_{0h})/100)$ and $[18:0m_{24h}] = ([\sum C18_{m}] \times P(18:0_{24h})/100)$

3) $[18:0B] = [18:0m_{24h}] - [18:0m_{0h}]$

Where,

$[\sum C18_{0h}]$ and $[\sum C18_{24h}]$, content (µg per tube) of total C18 FA in the 0 h and 24 h incubation tubes, respectively.

$[\sum C18_{m}]$, averaged content (µg per tube) of total C18 FA of both 0 h and 24 h incubation tubes.

$P(18:0_{0h})$ and $P(18:0_{24h})$, percentage of 18:0 in total C18 FA in the 0 h and 24 h tubes, respectively.

$[18:0_{m,0h}]$ and $[18:0_{m,24h}]$, content of 18:0 (µg per tube) expressed on $[\sum C18_{m}]$ basis.

$[18:0_{B}]$, balance (µg) of 18:0 during the incubation period (24 h – 0 h).

The biohydrogenation of dietary unsaturated C18 FA were then calculated as:

4) BH of FA (%) = $(([FA_{m,0h}] - [FA_{m,24h}])/ [FA_{m,0h}]) \times 100$

Where FA, can be *c*9-18:1, *c*9,*c*12-18:2 or *c*9,*c*12,*c*15-18:2 and BH stands for biohydrogenation.

All of C18 FA that displayed a positive balance during the 24 h of incubation, were considered here as biohydrogenation products (BHP). The relative yield of the main classes of BHP (18:0, 18:1 isomers; 18:2 isomers and oxo-FA) were computed from the C18 FA balance data and expressed in percentage of total BHP, as exemplified for 18:0:

5) $(18:0Y) = ([18:0_B] \times 100)/ [BHP_B]$

Where,

(18:0Y), relative yield of 18:0 expressed in percentage of total BHP

$[BHP_B]$, sum of all data of C18 FA

The ratios between BH of each dietary unsaturated C18 FA (*c*9-18:1, *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:2) and the total VFA (TVFA) were also computed.

### 5.2.6 Statistical analysis

Data were analyzed using the Proc Mixed of SAS (SAS Institute inc, Cary, NC, USA), considering the fixed effect of treatments and the 5 incubations runs as random block. Orthogonal contrasts were used to detect linear and quadratic responses. Least square means and standard error of the mean (SEM) are presented in tables. Differences were declared significant at $P<0.05$.

## 5. 3 Results

The net production of TVFA and individual VFA are presented in Table 5.1. Increasing CT doses caused a linear decrease in TVFA production ($P$ <0.001) and in all individual VFA production, except for iso-5:0, that decreased quadratically ($P$ = 0.004). However, the 2:0/3:0 ratio was not influenced by CT, averaging 3.0 mmol/L.

**Table 5.1.** Effect of different doses of *Cistus ladanifer* condensed tannins extract (0, 25, 50, 75 and 100 g/kg DM) on net production of volatile fatty acid (VFA, mmol/L) during the 24 h of incubation

| | *Cistus ladanifer* condensed tannins extract | | | | | SEM | *P* value | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 25 | 50 | 75 | 100 | | Linear | Quad. |
| Total VFA | 35.4 | 33.2 | 32.6 | 26.6 | 25.8 | 1.59 | <0.001 | 0.629 |
| 2:0 | 22.0 | 20.9 | 20.7 | 16.4 | 16.3 | 1.20 | <0.001 | 0.623 |
| 3:0 | 7.28 | 6.79 | 6.62 | 5.60 | 5.19 | 0.314 | <0.001 | 0.554 |
| iso-4:0 | 0.41 | 0.24 | 0.26 | 0.20 | 0.16 | 0.042 | <0.001 | 0.219 |
| 4:0 | 4.43 | 4.33 | 4.15 | 3.68 | 3.45 | 0.205 | <0.001 | 0.190 |
| iso-5:0 | 0.61 | 0.46 | 0.41 | 0.36 | 0.33 | 0.025 | <0.001 | 0.004 |
| 5:0 | 0.50 | 0.40 | 0.37 | 0.30 | 0.27 | 0.020 | <0.001 | 0.107 |
| 2:0/3:0 ratio | 3.04 | 3.09 | 3.015 | 2.92 | 3.13 | 0.138 | 0.734 | 0.738 |

Dietary unsaturated C18 FA BH are presented in Table 5.2. The BH of *c*9-18:1 and *c*9,*c*12-18:2 did not differ ($P > 0.05$) among treatments. *c*9,*c*12,*c*15-18:3 BH decreased linearly with CT increasing doses.

Condensed tannins had no effect on 18:0 and 18:1 yield. Yield of 18:0 ranged from 45.6 to 50.2 % of total BHP, and 18:1 BHP yield ranged 34.0 to 35.9% of total BHP. However, yield of 18:2 BHP decreased linearly with CT increasing doses. Yield of oxo-FA showed a quadratic increase being higher in treatments with CT (averaging 14.3%) than in control (11.5%).

The BH/VFA ratios, showed in Table 5.2, was calculated in order to evaluate the relation between BH and fermentative activity. The BH/VFA ratios for *c*9-18:1 and *c*9,*c*12-18:2 increased linearly with CT dose, whereas BH/VFA ratio for *c*9,*c*12,*c*15-18:3 was unaffected by CT dose.

Table 5.3 shows the effect of CT increasing doses on C18 FA balance during the 24h of incubation. The *c*9-18:1; *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:3, the main dietary unsaturated C18 FA, displayed large negative balances (losses) in all treatments. The losses of *c*9-18:1; *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:3 did not differ among treatments ($P > 0.05$).

The C18 FA displaying positive balance (i.e. biohydrogenation products – BHP) comprise 18:0, 18:1 and 18:2 isomers and oxo-FA. Occasionally, other C18 FA (i.e. *c*11-18:1) presented negative balances, but the overall pattern was to present positive balances. Stearic acid (18:0) comprised 656 µg/tube to 774 µg/tube formed and did not differ significantly among the CT doses tested. All 18:1 isomers, except *c*11-18:1, displayed consistently positive balances that were larger for *t*11-18:1 comprising 324 µg/tube to 456 µg/tube and was not affected by CT. The *t*4-, *t*5-, *t*6-+*t*7-+*t*8-, *t*15- and *t*16+*t*14-18:1 isomers increased linearly with the increasing

CT doses, whereas the *t*9-18:1 isomer decreased linearly, and none of the other 18:1 isomers identified was significantly affected by *C. ladanifer* CT extract.

**Table 5.2.** Effect of different doses of *Cistus ladanifer* condensed tannins extract (0, 25, 50, 75 and 100 g/kg DM) on estimated biohydrogenation, relative yield in the biohydrogenation products (BHP) and biohydrogenation:fermentation ratio.

| | *Cistus ladanifer* condensed tannins extract | | | | | SEM | *P* values | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 25 | 50 | 75 | 100 | | Linear | Quad. |
| Biohydrogenation (%) | | | | | | | | |
| *c*9-18:1 | 51.2 | 52.4 | 47.1 | 48.6 | 48.5 | 4.30 | 0.426 | 0.760 |
| *c*9,*c*12-18:2 | 68.7 | 67.1 | 63.4 | 64.6 | 65.0 | 3.93 | 0.431 | 0.545 |
| *c*9,*c*12,*c*15-18:3 | 67.6 | 61.2 | 52.3 | 54.7 | 50.8 | 3.76 | 0.003 | 0.239 |
| Biohydrogenation Products (%) | | | | | | | | |
| 18:0 | 49.2 | 47.8 | 45.6 | 50.2 | 49.5 | 3.50 | 0.666 | 0.327 |
| 18:1 BHP | 35.8 | 35.3 | 35.9 | 34.0 | 35.0 | 4.65 | 0.553 | 0.952 |
| 18:2 BHP | 4.18 | 3.36 | 3.44 | 2.36 | 2.34 | 0.608 | 0.020 | 0.841 |
| oxo-FA | 11.5 | 14.1 | 15.4 | 13.9 | 13.6 | 2.15 | 0.046 | 0.001 |
| Biohydrogenation:Fermentation ratio | | | | | | | | |
| *c*9-18:1BH/VFA | 1.47 | 1.59 | 1.45 | 1.86 | 1.95 | 0.182 | 0.009 | 0.340 |
| *c*9,*c*12-18:2BH/VFA | 1.95 | 2.03 | 1.95 | 2.47 | 2.58 | 0.171 | 0.004 | 0.278 |
| *c*9,*c*12,*c*15-18:3BH/VFA | 1.93 | 1.85 | 1.60 | 2.10 | 2.01 | 0.159 | 0.437 | 0.235 |

Collectively, the 18:2 isomers comprised from 37 µg/tube (in 4.5 and 6% of CT) to 69 µg/tube of total BHP formed (in Control). The 18:2 isomers showed a quadratic response being highest in Control ($P = 0.018$) than in CT treatments. The major individual 18:2 isomer was the *t*9,*c*12-18:2, which ranged from 9.4 µg/tube to 21.5 µg/tube and presented a quadratic response ($P = 0.047$) similar to the 18:2 isomers sum. The *c*9,*t*11-, *t*9,*t*12- and *c*9,*t*12-18:2 decreased linearly with increasing CT doses. Relatively to oxo-FA, only the 13-oxo-18:0 increased linearly ($P = 0.019$) with CT dose whereas the others oxo-FA were unaffected.

Table 5.4 shows the effect CT increasing doses on the balance of other non-C18 FA, mostly derived from microbial *de novo* synthesis, and dimethylacetals (DMA) during the 24h of incubation. Mostly of these FA presented a positive balance, except the 12:0, 20:1 and *c*7-16:1. The 14:0, 16:0, cyclo-17:0 and 26:0 FA decreased linearly with CT doses, whereas, the 28:0 and *c*7-16:1 presented a quadratic response, with lower values for 3 and 4.5% CT in 28:0, and for 3% in *c*7-16:1.

All the odd- and branched-chain FA (OBCFA) were affected by CT and presented a similar trend, decreasing linearly with the CT dose, with exception for the 13:0 that showed a quadratic response, with higher value in the control (0% of CT) and lower in 4.5% of CT.

The total of DMA comprised from 255 to 721 µg/g of DM and presented a quadratic response to the CT dose. All DMA decreased linearly with CT dose, except for 18:1 that presented a quadratic response, with lower value in the 3% of CT.

**Table 5.3.** Effect of different doses of *Cistus ladanifer* condensed tannins extract (0, 25, 50, 75 and 100 g/kg DM) on net C18 fatty acid changes (µg/tube) during the 24 h of incubation.

| | *Cistus ladanifer* condensed tannins extract | | | | | SEM | *P* values | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 25 | 50 | 75 | 100 | | Linear | Quad. |
| C18 FA loss | | | | | | | | |
| *c*9-18:1 | -635 | -646 | -576 | -597 | -597 | 57.5 | 0.479 | 0.730 |
| *c*9,*c*12-18:2 | -1004 | -946 | -827 | -896 | -956 | 240.2 | 0.617 | 0.230 |
| *c*9,*c*12,*c*15-18:3 | -23.5 | -21.5 | -16.9 | -19.4 | -18.4 | 3.61 | 0.061 | 0.233 |
| Sum | -1663 | -1613 | -1420 | -1512 | -1572 | 259.4 | 0.544 | 0.364 |
| C18 FA gains | | | | | | | | |
| 18:0 | 774 | 750 | 656 | 743 | 753 | 100.1 | 0.836 | 0.380 |
| 18:1 isomers | | | | | | | | |
| *t*4- | 2.84 | 3.33 | 3.60 | 4.44 | 4.68 | 0.718 | <0.001 | 0.947 |
| *t*5- | 1.25 | 0.93 | 2.08 | 2.40 | 3.27 | 0.815 | 0.002 | 0.395 |
| *t*6-, *t*7-, *t*8- | 22.8 | 27.1 | 27.1 | 31.5 | 33.3 | 4.73 | 0.009 | 0.960 |
| *t*9- | 61.7 | 54.7 | 43.4 | 42.2 | 40.2 | 6.40 | <0.001 | 0.538 |
| *t*10- | 33.6 | 40.7 | 40.4 | 42.5 | 41.8 | 7.18 | 0.276 | 0.493 |
| *t*11- | 456 | 397 | 324 | 333 | 376 | 136.9 | 0.186 | 0.155 |
| *t*12- | 27.4 | 29.8 | 28.6 | 32.2 | 33.7 | 4.79 | 0.131 | 0.780 |
| *t*15- | 14.3 | 16.4 | 15.1 | 19.0 | 20.7 | 3.08 | 0.025 | 0.566 |
| *c*11- | -10.6 | -11.5 | -8.1 | -10.0 | -9.4 | 2.90 | 0.518 | 0.747 |
| *c*12- | 22.2 | 21.2 | 18.5 | 20.0 | 21.2 | 4.23 | 0.695 | 0.391 |
| *c*13- | 1.16 | 1.05 | 0.35 | 1.75 | 1.34 | 0.289 | 0.268 | 0.180 |
| *t*16-, *t*14- | 12.5 | 15.8 | 14.3 | 16.9 | 18.3 | 2.75 | 0.028 | 0.951 |
| *c*16- | 2.99 | 3.29 | 3.09 | 2.92 | 3.39 | 0.678 | 0.634 | 0.736 |
| Sum | 654 | 607 | 522 | 544 | 593 | 153.2 | 0.371 | 0.227 |
| 18:2 isomers | | | | | | | | |
| *t*9,*t*12- | 9.47 | 6.33 | 5.34 | 4.18 | 3.47 | 0.677 | <0.001 | 0.080 |
| *c*9,*t*12- | 17.1 | 13.4 | 11.0 | 8.7 | 6.9 | 2.33 | <0.001 | 0.543 |
| *t*11,*c*15- | 1.27 | 1.33 | 1.07 | 1.11 | 0.62 | 0.343 | 0.101 | 0.437 |
| *c*9,*t*11- | 8.02 | 3.83 | 0.26 | 0.57 | 1.39 | 2.178 | 0.022 | 0.092 |
| CLA *tt* | 12.3 | 12.7 | 10.8 | 11.9 | 14.9 | 3.18 | 0.536 | 0.336 |
| *t*9,*c*12- | 21.5 | 15.4 | 12.9 | 10.3 | 9.4 | 2.80 | <0.001 | 0.047 |
| Sum | 69.0 | 53.0 | 41.4 | 36.8 | 36.5 | 8.83 | <0.001 | 0.018 |
| Oxo FA | | | | | | | | |
| 10-oxo-18:0 | 147 | 180 | 175 | 163 | 162 | 17.9 | 0.819 | 0.280 |
| 13-oxo-18:0 | 24.1 | 30.6 | 30.0 | 30.8 | 31.8 | 5.09 | 0.019 | 0.192 |
| Sum | 171 | 210 | 205 | 194 | 194 | 19.9 | 0.354 | 0.273 |
| Total gain | 1663 | 1613 | 1420 | 1512 | 1572 | 259.4 | 0.544 | 0.364 |

**Table 5.4.** Effect of different doses of *Cistus ladanifer* condensed tannins extract (0, 25, 50, 75 and 100 g/kg DM) on other fatty acids (FA) and dimethylacetals (DMA) balance (µg/g DM) (difference between 24 and 0 h).

| | *Cistus ladanifer* condensed tannins extract | | | | | SEM | *P* values | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 25 | 50 | 75 | 100 | | Linear | Quad. |
| FA | | | | | | | | |
| 12:0 | -99.8 | -96.7 | -146.4 | -79.7 | -102.9 | 34.67 | 0.921 | 0.619 |
| 14:0 | 190 | 181 | 85.3 | 98.6 | 62.0 | 26.6 | <0.001 | 0.591 |
| 16:0 | 1456 | 1274 | 316 | 785 | 329 | 347.1 | 0.021 | 0.507 |
| cyclo-17:0 | 37.0 | 54.9 | 23.2 | 10.4 | -10.2 | 13.10 | 0.003 | 0.252 |
| 20:0 | 50.6 | 47.3 | 13.3 | 37.4 | 22.4 | 13.69 | 0.141 | 0.207 |
| 20:1 | -7.38 | -6.85 | -18.81 | -10.68 | -11.31 | 4.524 | 0.423 | 0.307 |
| 21:0 | 0.94 | 3.03 | 0.34 | -0.80 | 3.64 | 1.32 | 0.707 | 0.213 |
| 22:0 | 43.9 | 37.1 | -3.93 | 21.0 | 14.0 | 15.99 | 0.149 | 0.056 |
| 23:0 | 9.74 | 5.80 | 2.30 | 6.50 | 4.99 | 2.708 | 0.316 | 0.229 |
| 24:0 | 39.1 | 34.3 | 10.4 | 25.5 | 10.4 | 10.03 | 0.050 | 0.629 |
| 26:0 | 164 | 112 | 72.0 | 87.5 | 69.1 | 17.32 | <0.001 | 0.073 |
| 28:0 | 8.38 | 10.79 | 0.44 | 0.06 | 10.49 | 3.151 | 0.521 | 0.039 |
| 16:1c7 | -1.77 | -1.45 | -11.82 | -5.74 | -5.67 | 4.276 | 0.063 | 0.041 |
| 17:1c9 | 1.51 | -0.80 | -2.24 | 1.69 | -2.36 | 2.006 | 0.417 | 0.805 |
| OBCFA | | | | | | | | |
| 13:0 | 15.8 | 7.50 | 9.09 | 4.30 | 7.48 | 2.093 | 0.005 | 0.036 |
| i-14:0 | 28.9 | 26.6 | 19.9 | 16.1 | 14.0 | 5.33 | 0.027 | 0.879 |
| i-15:0 | 64.4 | 45.5 | 24.7 | 21.6 | 9.4 | 5.06 | <0.001 | 0.062 |
| a-15:0 | 139 | 124 | 75.1 | 71.2 | 50.7 | 9.13 | <0.001 | 0.334 |
| 15:0 | 130 | 111 | 58.0 | 61.6 | 43.8 | 13.5 | <0.001 | 0.251 |
| i-16:0 | 44.2 | 47.8 | 32.2 | 26.7 | 20.2 | 5.36 | <0.001 | 0.558 |
| i-17:0 | 46.8 | 35.8 | 22.7 | 30.6 | 18.6 | 5.36 | 0.001 | 0.322 |
| a-17:0 | 72.5 | 58.1 | 38.0 | 42.3 | 20.8 | 9.61 | <0.001 | 0.739 |
| 17:0 | 73.4 | 52.3 | 31.4 | 33.8 | 23.5 | 10.14 | 0.001 | 0.228 |
| Total | 615 | 508 | 311 | 308 | 209 | 52.4 | <0.001 | 0.301 |
| DMA | | | | | | | | |
| 12:0 | 16.2 | 17.2 | 11.1 | 6.46 | 2.93 | 2.774 | <0.001 | 0.327 |
| i-14:0 | 18.5 | 13.3 | 0.63 | 3.00 | -2.44 | 3.587 | <0.001 | 0.254 |
| 14:0 | 31.0 | 23.3 | 11.3 | 5.47 | -6.31 | 5.258 | <0.001 | 0.911 |
| i-15:0 | 10.2 | 13.1 | 5.94 | 3.58 | -0.41 | 2.528 | <0.001 | 0.300 |
| a-15:0 | 528 | 392 | 260 | 183 | 221 | 54.79 | <0.001 | 0.051 |
| 15:0 | 9.53 | 8.36 | 2.64 | 1.83 | 0.51 | 2.235 | 0.002 | 0.588 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 16:0 | 68.5 | 55.9 | 40.7 | 37.0 | 18.4 | 6.28 | <0.001 | 0.983 |
| 18:0 | 9.80 | 4.18 | 4.59 | 3.28 | 3.80 | 1.864 | 0.018 | 0.087 |
| 18:1 | 29.3 | 19.3 | 6.46 | 18.4 | 16.8 | 3.45 | 0.020 | 0.003 |
| Total | 721 | 546 | 343 | 262 | 255 | 58.6 | <0.001 | 0.041 |

## 5. 4 Discussion

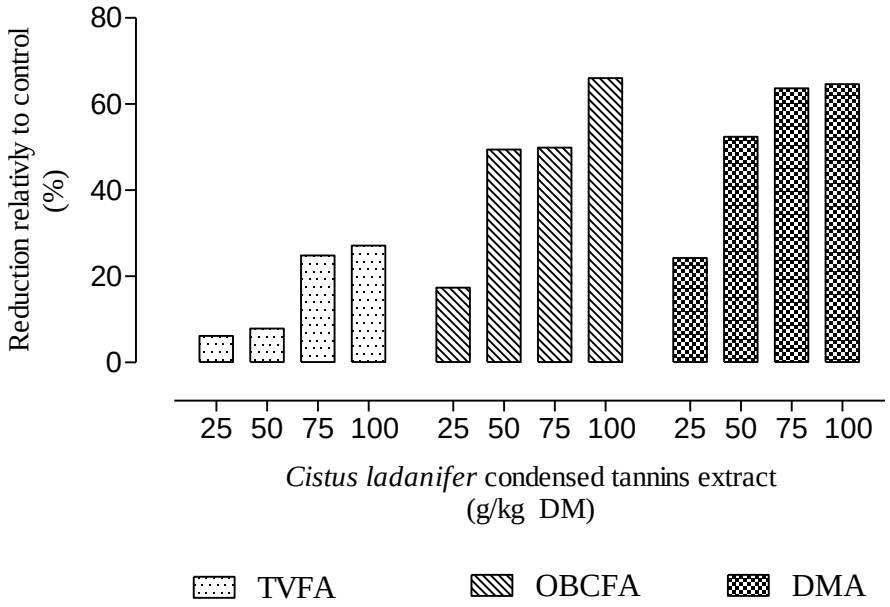
Utilization of CT extracts from various origins to modulate the ruminal BH, as a way to increase the rumen outflow of healthy FA has been the subject of several studies. Although, some results have shown the potential of this strategy to improve the FA profile of ruminant edible fat, overall the effects of CT extracts on ruminal metabolism including fermentation (Foiklang, Wanapat, & Norrapoke, 2016; Frutos, Hervas, Giraldez, & Mantecon, 2004; Guerreiro *et al.*, 2016a; Hatew *et al.*, 2016) and BH (Carreño *et al.*, 2015; Costa *et al.*, 2017a; Guerreiro *et al.*, 2016a; Khiaosa-Ard *et al.*, 2009; Vasta *et al.*, 2009a) are inconsistent. The diversity of CT extracts effects on ruminal fermentation and BH may be due to several factors, such as CT composition and concentration, basal substrate, and experimental conditions. In the current study, the increasing levels of *C. ladanifer* CT extract reduced the total and individual VFA production, which is in disagreement with previous *in vitro* studies where 100 g/kg DM of *C. ladanifer* CT extract did not depress ruminal fermentation (Costa *et al.*, 2017a; Guerreiro *et al.*, 2016a; Jerónimo *et al.*, 2010). Effects on ruminal BH was also inconsistent with previous *in vitro* studies with *C. ladanifer* CT extracts, where incubation of 100 g/kg DM of extract led to increase of *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:2 BH and increased of *t*11-18:1 production (Costa *et al.*, 2017a; Guerreiro *et al.*, 2016a). Conversely in present work, incubation of *C. ladanifer* CT extract at levels between 25 to 100 g/kg DM had limited effect on BH, affecting only *c*9,*c*12,*c*15-18:3 BH that decreased and the production of the minor BHP, without change of *t*11-18:1 production. *Cistus ladanifer* CT extract used in present work was obtained from the same *C. ladanifer* plants and by the same extraction methodology used in Guerreiro *et al.*, (2016a), the tested doses were same or below to the dose used previously (Costa *et al.*, 2017a; Guerreiro *et al.*, 2016a). However, in present work was used different incubation conditions relatively to previous *in vitro* works (Costa *et al.*, 2017a; Guerreiro *et al.*, 2016a), specifically in the basal substrate, buffer solution and incubation time, which may help to explain the difference of results on fermentation and BH.

Odd- and branched-chain FA (OBCFA) in rumen are mainly present in membrane lipids of rumen bacteria, which are able to synthetize them from propionate and/or branched-chain VFA derived from branched-chain aminoacids, and incorporate into their cell membranes (Kaneda,

1991). Rumen bacterial lipids are also characterized by high levels of plasmalogens (Miyagawa, 1982), a subclass of phospholipids containing alk-1-enyl (vinyl) ether chains which can be detected by the presence of DMA released under acid catalysis (Alves *et al.*, 2013b). Thus, OBCFA and DMA have been appointed as tools for prediction of the rumen fermentation pattern and as microbial markers in rumen ecosystems (Alves *et al.*, 2013b; Bessa *et al.*, 2009; Vlaeminck *et al.*, 2006). The general decrease of OBCFA and DMA production with increasing levels of *C. ladanifer* CT extract associated with reduction of VFA production suggest a reduction of bacterial activity. In fact, CT has inhibitory effect on activity and growth of microorganisms, which is probably due to their ability to form complexes with various types of molecules such as proteins, polysaccharides and minerals, allowing them to interact with microorganism's membranes, cell walls and extracellular structures and also reduce nutrient availability (Smith *et al.*, 2005).

The inhibitory effects of *C. ladanifer* CT extract were more pronounced for OBCFA and DMA production (up to 65% of reduction with 100 g/kg DM of *C. ladanifer* CT extract), than for the total VFA production (27% of reduction with 100 g/kg DM of *C. ladanifer* CT extract) (Figure 5.1). Apparently, the bacterial activities were differentially affected by *C. ladanifer* CT extract, with more pronounced effect on the anabolic activity than on the fermentative activity. Moreover, BH of dietary C18 unsaturated FA was little affected by increasing levels of *C. ladanifer* CT extract, except for the decrease in disappearance of the minimal amounts of *c*9,*c*12,*c*15-18:3. Thus, it is apparent that there was an uncoupling between BH activity and other microbial metabolic activities, and within the later between energy yielding catabolism (i.e. VFA production) and microbial growth (i.e. OBCFA and DMA production). Polyunsaturated fatty acids (PUFA) has well established bacteriostatic effects and it is reported that *Butyrivibrio fibrisolvens* growth was initiated only when PUFA (*c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:3) had been metabolized and reduced to *t*11-18:1 (Maia *et al.*, 2010). In fact, ruminal BH has been proposed to be a mechanism to minimizing its direct effects of PUFA on bacterial membranes stability by reduce the concentration of the PUFA in the rumen (Maia *et al.*, 2007). Thus, maintenance of high BH activity compared to grow and fermentative activity might reflect the need to first neutralize toxic effects of PUFA. In addition to the need to solve effects of PUFA, CT may also have induced a response of microbial population as a way of adjusting to high CT levels.

**Figure 5.1.** Effect of different doses of *Cistus ladanifer* condensed tannins extract (25, 50, 75 and 100 g/kg) on the reduction of total volatile fatty acids (TVFA), odd- and branched-chain fatty acids (OBCFA) and dimethylacetals (DMA), relatively to control




Low microbial growth efficiency in rumen is explained by use of part of the available energy for non-growth functions, as maintenance and energy spilling (Hackmann & Firkins, 2015). The microbial maintenance functions include: i) motility, ii) turnover and iii) maintenance of ion gradients across the cell membrane, and the maintenance costs is proportionally higher when fermentation and growth rates are slow (Russell, 2007). Condensed tannins can affect membrane integrity of microorganisms (Smith *et al.*, 2005). However, it is reported that bacteria are able to modify the membrane FA composition as response to environmental stimuli, in order to adjust its membrane fluidity. As defense mechanism against toxic effect, some bacteria reduce the membrane fluidity by incorporation of *trans*-FA into their membranes (Keweloh & Heipieper, 1996). Moreover, has been proposed that one the main goal of rumen bacterial BH may be related with production of the *trans*-FA and their incorporation into microbial cell as protective mechanism against toxicity of PUFA (Bessa *et al.*, 2000). So, in response to adverse condition promoted by high levels of CT, the microbial population may have developing an adaptive response in order to maintain the physiological properties of membrane, limiting the microbial growth. On the other hand, energy spilling refers to energy dissipation when the catabolic rate is faster than the anabolic rate (Russell, 2007). Energy spilling is appointed as the cause for low microbial growth efficiency during carbohydrate excess, but also in other conditions that limit the growth (Hackmann & Firkins, 2015). So, the reduction of microbial growth associated to limited effect on fermentation and maintenance of the BH activity

observed in present study is concordant with increase of the energy spilling, as consequence of the inhibitory effect of CT on microbial growth.

The ratio between BH of dietary C18 FA (*c*9-18:1; *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:3) and TVFA can be an indicator of BH activity relatively to fermentation activity. The ratio increased for *c*9-18:1 and *c*9,*c*12-18:2 with increasing levels of *C. ladanifer* CT extract, while ratio for *c*9,*c*12,*c*15-18:3 remained unchanged. Such result suggests an increment of BH activity relatively to fermentation in incubations with highest levels of *C. ladanifer* CT extract. Basal substrate was supplemented with 6% of sunflower oil, which shown as main FA the *c*9,*c*12-18:2, followed by *c*9-18:1, while *c*9,*c*12,*c*15-18:3 is present in lower levels. The increment of BH activity associated to increase of *C. ladanifer* CT extract might result of the additive effect of both stress stimuli on microbial population, in order to increase the *trans*-FA production for incorporation into cell membranes as protection against toxic effect of tannins.

Two oxo-FA, that are associated to BH of dietary C18 FA, were identified in ruminal fluid, representing both 10-14% of the total BHP products. The 10-oxo-18:0 and 13-oxo-18:0 has been described as end products of the BH of the *c*9-18:1 and *c*9,*c*12-18:2, respectively (Alves *et al.*, 2013b; Guerreiro *et al.*, 2016a; Jenkins *et al.*, 2006; Kairenius *et al.*, 2011; McKain *et al.*, 2010). The presence of both oxo-FA in ruminal fluid is concordant with high levels of *c*9-18:1 and *c*9,*c*12-18:2 in basal substrate. Increasing levels of *C. ladanifer* CT extract led to increase of the 13-oxo-18:0 production, while the production of 10-oxo-18:0 remained unchanged. Moreover, increasing levels of *C. ladanifer* CT extract also led to reduction of the production of some 18:2 BHP associated to BH of *c*9,*c*12-18:2, as *c*9,*t*12-18:2 and *c*9,*t*11-18:2, suggesting the occurrence of shift in the rumen microbiota composition favoring the production of the 13-oxo-18:0. Indeed, tannins is able to change the ruminal population composition (Smith *et al.*, 2005).

Although, *C. ladanifer* CT extract had induced to a strong inhibition on growth, the microbial population appear to be capable to overcome to stress condition induced by high levels of CT, maintaining the fermentation and BH activities. In fact, it is described the adaptation of the ruminal population to diets rich in CT (Smith *et al.*, 2005). So, the results observed in present work might be a first step to ruminal population adaptation to diets rich in CT.

## 5. 5 Conclusion

Increasing levels of *C. ladanifer* CT extract had minor effect on the BH, without change the *t*11-18:1 production. However, *C. ladanifer* CT extract resulted in reduction of the VFA production and in very pronounced depression of OBCFA and DMA production. The increasing levels of *C. ladanifer* CT extract appear inhibit the microbial growth, without affect the fermentative and BH activities, which may constitute an adaptive response of microbial population to high levels of CT. Present results, also emphasize the inconsistency regarding effects of CT on rumen fermentation and BH. Further studies are needed to elucidate the impact of CT on ruminal population and metabolism, for adequate use of CT extract as feed additive to improve the nutritional value of ruminant fat

## Acknowledgements

The authors would like to thank Alentejo2020 program for financial support to the project "CistusRumen - Sustainable use of Rockrose (*Cistus ladanifer* L.) in small ruminants - Increase of the competitiveness and reduction of the environmental impact" (ALT20-03-0145-FEDER-000023) through the Regional Development European Fund. We also thank the Fundação para a Ciência e Tecnologia (FCT) through research grants to OG (SFRH/BD/84406/2012), MC (SFRH/BD/90468/2012) and SPA (SFRH/BPD/76836/2011) and through the PTDC/CVT/120122/2012 and UID/CVT/00276/2013 projects.

# CHAPTER 6

## INCLUSION OF AERIAL PART AND CONDENSED TANNINS EXTRACT FROM *CISTUS LADANIFER* L. ON LAMB DIET – EFFECT ON GROWTH PERFORMANCE, CARCASS AND MEAT QUALITY AND FATTY ACID COMPOSITION OF INTRAMUSCULAR AND SUBCUTANEOUS FAT

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Article in advanced preparation to be submitted to *Meat Science*

## Abstract

Thirty six lambs were used to evaluate the effect of two levels of *C. ladanifer* condensed tannins (CT) (1.25% and 2.5%) and two ways of CT supply (*C. ladanifer* aerial parts and *C. ladanifer* CT extract) on lamb growth performance, carcass composition, meat quality and FA composition of intramuscular and subcutaneous fat. Moreover, a diet without CT sources was also tested, and all diets were supplemented with soybean oil (60 g/kg). Lambs stayed on trial for 5 weeks until slaughter. The highest amounts of CT (2.5%) had detrimental effects on growth performance, particularly when was used the *C. ladanifer* aerial part, without beneficial effect on FA composition of intramuscular and subcutaneous fat. Inclusion of 1.25% of CT from *C. ladanifer* extract in diets increased the *t*11-18:1 in intramuscular and subcutaneous fat, but not affect the *c*9,*t*11-18:2.

**Keywords**: *Cistus ladanifer* L.; condensed tannins; fatty acids; lamb meat; carcass quality

## 6. 1 Introduction

Nutritional recommendations have been promoted a reduction of the fat consumption and replacement of the saturated fatty acids (SFA) by healthy fatty acids (FA) in diets, to limit the development of the several chronic diseases, as cardiovascular diseases. So, due to its high levels of saturated fatty acids (SFA) and low content of polyunsaturated fatty acids (PUFA), the consumption of ruminant fat has been associated to detrimental effect on human health. In the rumen, dietary lipids are rapidly hydrolysed and released unsaturated FA are biohydrogenated, with production of high levels of SFA as well as a variable amount of unsaturated FA, mostly of them *trans* FA, like *trans* 18:1 and conjugated and non-conjugated isomers of linoleic acid (18:2*n*-6), as result of the not complete biohydrogenation (BH) of unsaturated FA (Harfoot & Hazlewood, 1997). Health implications of mostly these *trans* FA are yet unknown, but vaccenic (*t*11-18:1) and rumenic acids (*c*9,*t*11-18:2) are look as beneficial to human health (Kuhnt *et al.*, 2016). Rumenic acid is the main conjugated linoleic acid isomers (CLA) present in ruminant fat and diverse *in vitro* and animal models studies demonstrated that has anti-inflammatory, anti-atherogenic, anti-obese and anti-carcinogenic activities (Kuhnt *et al.*, 2016; Shokryzadan *et al.*, 2017). Rumenic acid is a biohydrogenation intermediate (BI) of 18:2*n*-6, but its main via of synthesis is through desaturation of *t*11-18:1 by stearoyl-CoA desaturase (SCD) in tissues and mammary gland (Griinari *et al.*, 2000; Palmquist *et al.*, 2004). Nutritional strategies to enrich ruminant fat with PUFA, *t*11- 18:1 and *c*9,*t*11-18:2 had been extensively researched, and supplementation of diets with plants or plants extracts rich in secondary compounds like polyphenolic compounds seems to be a promising approach.

*Cistus ladanifer* L. is a perennial shrub quite abundant in Mediterranean region, that contain low levels of protein, low digestibility of organic matter and high levels of phenolic compounds, as condensed tannins (CT) (Dentinho *et al.*, 2005; Guerreiro *et al.*, 2016b). Despite its poor nutritional value and the presence of anti-nutritional factors, *C. ladanifer* aerial part is consumed by ruminant species in Mediterranean grassing systems, constituting probably an important feeding resource, along with other shrubs and tree foliage, as a complement to pasture and commercial concentrates. As far we know, the use of *C. ladanifer* aerial part in ruminant diets was only tested in three productive experiments with lambs, with incorporation levels between 50 and 250 g *C. ladanifer*/kg (Francisco *et al.*, 2017; Francisco *et al.*, 2015; Jerónimo *et al.*, 2010). Independently the *C. ladanifer* levels in diets, animal performance was not compromised (Francisco *et al.*, 2017; Francisco *et al.*, 2015; Jerónimo *et al.*, 2010). Moreover, these studies revealed the capacity of *C. ladanifer* to modulate the ruminal BH (Francisco *et al.*, 2015; Jerónimo *et al.*, 2010). When incorporated into a diet of dehydrated Lucerne

supplemented with PUFA-rich vegetable oils, *C. ladanifer* led to increase of *t*11-18:1 on abomasal digesta and to increase of *t*11-18:1 and *c*9,*t*11-18:2 on intramuscular fat (Jerónimo *et al.*, 2010). These results were associated to the high amounts of CT present in *C. ladanifer* (Jerónimo *et al*., 2010), which came to be supported by an *in vitro* study, where it was verified that CT extract is the most active fraction of *C. ladanifer* to modulate rumen BH, inducing the increase of BH of *c*9,*c*12-18:2 and *c*9,*c*12,*c*15-18:3 and the accumulation of *t*11-18:1 and *c*9,*t*11-18:2 (Guerreiro *et al.*, 2016a).

Thus, we hypothesized that incorporation of CT extract from *C. ladanifer* in lamb diets might induce the same effect as the use of *C. ladanifer* aerial plant on FA profile of intramuscular fat, i.e. increase of *t*11-18:1 and *c*9,*t*11-18:2. Moreover, inclusion levels of *C. ladanifer* CT in diets that allows optimizing the levels of *t*11-18:1 and *c*9,*t*11-18:2 in ruminant fat is not yet known. Therefore, the present experiment was designed to explore the effect of two levels of *C. ladanifer* CT (1.25% and 2.5%) and two ways of CT supply (leaves and soft stems of *C. ladanifer* and *C. ladanifer* CT extract) on lamb growth performance, carcass composition, meat quality and FA composition of intramuscular fat and subcutaneous fat.

## 6. 2 Material and methods

### 6.2.1 Animals, diets and management

The experiment was conducted at the facilities of Polo de Investigação da Fonte Boa, Instituto Nacional de Investigação Agrária e Veterinária (INIAV), Vale de Santarém, Portugal and the animal handling followed EU Directive 2010/63/UE concerning animal care. Thirty-six crossbred Merino Branco x Romane ram lambs of approximately 60 days of age and with an average body weight of 19.9 ± 1.86 kg (mean ± s.d.) were randomly assigned to individual pens. Four diets were formulated considering two levels of *C. ladanifer* CT (1.25 and 2.5% of CT) and two ways of *C. ladanifer* CT supply (*C. ladanifer* leaves and soft stems *vs*. *C. ladanifer* CT extract). Moreover, a diet without CT sources was also prepared. So, the five diets were: (1) L – basal diet composed of dehydrated Lucerne; (2) CL1.25 – basal diet with 125 g of *C. ladanifer* /kg; (3) CL2.5 – basal diet with 250 g *C. ladanifer* /kg; (4) Ex1.25 – basal diet with 20.5 g of *C. ladanifer* CT extract/kg; and (5) Ex2.5 – basal diet with 41 g of *C. ladanifer* CT extract/kg. All diets were supplemented with soybean oil (60 g/kg). Six pens were randomly assigned to each diet with CT (CL1.25, CL2.5, Ex1.25 and Ex2.5), and twelve pens to L diet.

Leaves and soft stems of *C. ladanifer*, either to incorporate in CL1.25 and CL2.5 diets or to prepare *C. ladanifer* CT extract for the Ex1.25 and Ex2.5 diets, were harvested in southern of Portugal (37°43'33.5"N 8°21'42.5"W) between October 2016 and January 2017. For both uses, leaves and soft stems of *C. ladanifer* were dried at room temperature and milled to a final particle size of 1 mm. For preparation of *C. ladanifer* CT extract, ground *C. ladanifer* was added to acetone:water solution (70:30, v/v) at 1:5 of solid:liquid ratio, and the mixture maintained at 25 ºC for 24 h, with gentle agitation (Orbital shaker incubator, TEQ, Massamá, Portugal). The supernatant containing CT were recovered by filtration through four layers of gauze and pressed. To the filtrate was added the same amount of ground *C. ladanifer* and acetone:water solution (70:30, v/v) to reach the 1:5 of solid:liquid ratio. After 24 h at 25 ºC with gentle agitation, the supernatant was filtered through four layers of gauze and pressed. After acetone evaporation, extracts were frozen at -80 ºC and then freeze-dried (ScanVac CoolSafe, LaboGene ApS, Lynge, Denmarck). Condensed tannins content of *C. ladanifer* aerial part and *C. ladanifer* CT extract was 100.4 and 616.3 g/kg, respectively. For diets preparation, the dehydrated Lucerne was milled to a final particle size of 1 mm and mixed with *C. ladanifer* aerial part or *C. ladanifer* CT extract and with other feed ingredients. Ingredients and chemical composition of diets are presented in Table 6.1.

The trial started after an adaptation period of 7 days to experimental conditions and lasted for 35 days. Feed was offered daily at morning at the rate of 110% of *ad libitum* intake calculated by daily weighing of the fed refused in each pen. At the days 1, 8, 18, 28 and 35 of trial the lambs were weighed just before feeding.

### 6.2.2 Slaughter, carcass evaluation and sample collection

At the end of experimental period, lambs were transported to the experimental abattoir of INIAV, where they were stunned and slaughtered by exsanguination. After preparation, carcasses were immediately weighed to obtain hot carcass weight and were kept at 10 ºC for 24 h. After this period carcasses were re-weighed, to obtain the cold carcass weight and then chilled at 2 ºC for 48h.

On the third day after slaughter, kidney knob channel fat (KKCF) and kidneys were removed. Carcasses were split along the spine and left sides were separated into eight joints as described in Santos-Silva et al. (2002). The weight of each joint was recorded to estimate the proportion of the higher-priced joints (leg + chump + loin + ribs). Shoulders were vacuum-packed and frozen at -20 ºC until being dissected into muscle, subcutaneous and intermuscular fats and bone to determine the tissue composition.

**Table 6.1.** Ingredients, chemical composition and fatty acid (FA) profile of the experimental diets

| Item | Diets | | | | |
|---|---|---|---|---|---|
| | L | CL1.25 | CL2.5 | Ex1.25 | Ex2.5 |
| Ingredients (g/kg DM) | | | | | |
| Dehydrated lucerne | 918 | 793 | 668 | 897.5 | 877 |
| *Cistus ladanifer* (CL) | - | 125 | 250 | - | - |
| *C. ladanifer* condensed tannins extract (Ex) | - | - | - | 20.5 | 41 |
| Soybean oil | 60 | 60 | 60 | 60 | 60 |
| Sodium bicarbonate | 5 | 5 | 5 | 5 | 5 |
| Calcium carbonate | 13 | 13 | 13 | 13 | 13 |
| Salt | 4 | 4 | 4 | 4 | 4 |
| Chemical composition (g/kg DM) | | | | | |
| Dry matter (DM)[a] | 916 | 915 | 918 | 917 | 914 |
| Crude protein | 162 | 147 | 133 | 154 | 155 |
| NDF | 447 | 441 | 411 | 440 | 434 |
| ADF | 326 | 322 | 301 | 326 | 315 |
| ADL | 62 | 64 | 62 | 63 | 64 |
| Ash | 113 | 110 | 104 | 115 | 112 |
| Total phenols[b] | 5.75 | 12.7 | 23.0 | 10.9 | 15.8 |
| Condensed tannins[c] | 4.08 | 15.3 | 26.6 | 11.9 | 20.3 |
| Ether extract | 77.0 | 82.7 | 98.3 | 80.0 | 70.0 |
| Total fatty acids | 88.4 | 95.6 | 84.4 | 81.5 | 80.0 |
| 16:0 | 11.7 | 12.6 | 10.5 | 10.6 | 10.9 |
| 18:0 | 3.51 | 3.89 | 3.43 | 3.24 | 3.22 |
| *c*9-18:1 | 19.0 | 20.2 | 17.9 | 17.4 | 16.7 |
| 18:2*n*-6 | 44.7 | 47.4 | 42.0 | 41.1 | 39.6 |
| 18:3*n*-3 | 6.56 | 7.36 | 6.28 | 6.12 | 6.31 |
| Fatty acids (g/kg total fatty acids) | | | | | |
| 16:0 | 133 | 132 | 125 | 131 | 137 |
| 18:0 | 39.8 | 40.7 | 40.7 | 39.7 | 40.3 |
| *c*9-18:1 | 215 | 212 | 213 | 214 | 209 |
| 18:2*n*-6 | 505 | 495 | 497 | 505 | 496 |
| 18:3*n*-3 | 74.2 | 76.9 | 74.4 | 75.1 | 79.0 |

[a] g/kg feed, [b] Tannic acid equivalents, [c] Condensed tannins quantified using purified *Cistus ladanifer* CT as standard

In the left halves of carcasses, the rib joints containing the *Longissimus lumborum* (LL) muscle, were vacuum-packed and frozen at -20 ºC until shear force determination. The *Longissimus thoracis* (LT) muscle was removed from loin joints, and the color was determined after 1 h of blooming. After removal of the epimysium, one portion of LT was minced with a food processor (Moulinex-123 A320R1) (3 x 5s), vacuum-packed and stored at -20 ºC until lipid analysis and pH determination.

From the right halves, subcutaneous fat was collected and then stored at -20 °C for FA analysis. The right loin joints containing *Longissimus* muscle were vacuum packed and stored at -20°C until sensory analysis.

### 6.2.3 Feed and muscle chemical analysis

Diets were analyzed for DM content (ISO 6496, 1999b), ashes (ISO 5484, 2002), crude protein (ISO 5983, 1997), and ether extract (ISO 6492, 1999a). Neutral detergent fiber (NDF), assayed with sodium sulfite, without α-amylase and expressed with residual ash, and acid detergent fiber (ADF) were determined according to Van Soest *et al*. (1991). The extraction of phenolic compounds was carried out as described by Julkumen-Tiitto (1985) and Makkar (2003b). The extracts obtained were used for the determination of total phenols and CT. Total phenols were determined using the Folin-Ciocalteu's assay, according to Guerreiro *et al*. (2016b), using tannic acid as standard and results expresses as tannic acid equivalents (TAE). Condensed tannins were determined using a butanol-HCl method, according to Porter *et al*. (1986). The concentration of CT was quantified using *C. ladanifer* purified CT as standard. Fatty acid methyl esters (FAME) of feed lipids were prepared by one-step extraction transesterification with toluene and nonadecanoic acid (19:0) as internal standard, according to Sukhija & Palmquist (1988).

Muscle and subcutaneous fat lipids were extracted with dichloromethane:methanol (2:1, v/v) from freeze-dried tissue samples and transesterified into FAME using a combined basic and acid catalysis as described by Oliveira *et al*. (2016).

Fatty acid methyl esters of feed, intramuscular and subcutaneous fat lipids were analyzed by gas chromatography coupled with flame ionization detection (GC-FID) (Shimadzu GC-2010 Plus, Kyoto, Japan), using a 100% cyanopropyl polysiloxane capillary column (SP-2560, 100 m, 0.25 mm i.d., 0.20 µm film thickness; Supelco Inc., Bellefont, PA, USA). Nonadecanoic acid (19:0) was used as internal standard for FA quantification. Identification of FAME was achieved by comparison of retention times with those of commercial standard mixtures (FAME

mix 37 components from Supelco Inc.), and by comparison with published chromatograms (Alves *et al.*, 2013a). Additional identification of the FAME was confirmed by electron impact MS using a Shimadzu GC-MS QP2010 Plus (Shimadzu, Kyoto, Japan) with a SP-2560 colunm. The chromatographic conditions for GC-FID and GC-MS were described in detail by Costa *et al*. (2017b).

### 6.2.4 Meat colour, pH, shear force and cooking loss

Meat colour was measured using a Minolta CR-300 chromometer (Konica Minolta, Lisboa, Portugal) according to CIE $L^*$, $a^*$, $b^*$ system, where $L^*$ is lightness, $a^*$ redness and $b^*$ yellowness. Three measurements per sample were recorded. Measurements were made using the C illuminant and 2º standard observers. Hue angle ($H^*$) was calculated as $\tan^{-1}(b^*/a^*)$ x $(180/\pi)$ and colour saturation (chroma, $C^*$) as $(a^{*2} + b^{*2})^{1/2}$. The pH was measured in a suspension of minced meat of LT in a solution of potassium chloride 0.1 M (1:10, w/v), using a pH meter (Metrohm 744) equipped with a combined glass electrode. For shear force determinations, the frozen loin samples were thawed for 24 h at 2 ± 1 °C. Thereafter, the *Longissimus* muscle was isolated, weighed, and cooked in an electric oven at 170 °C until the internal temperature reached 70 °C, which was monitored with an internal thermocouple (Thermometer, Omega RDXL4SD, Manchester, USA). Samples were cooled in a refrigerator at 4 °C during 24 h and then weighted for determination of the cooking loss, by difference of samples weight before and after cooking. Cooking loss was expressed as percentage of the initial weight. After cooling for 24 h at 2 ± 1 °C, samples were longitudinally cut in the direction of fibers into subsamples with a 1 $cm^2$ and with 2 cm long. Shear force was determined using a Warner–Bratzler shear device mounted in a Texture Analyzer (TA-tx2i Texture Analyzer; Stable Micro Systems, Godalming, UK) and equipped with a compression load cell of 25 kg. Samples were shear perpendicularly to the axis of the fibre direction using a crosshead speed of 2 mm/s, along 25 mm, according to procedures described by Francisco *et al*. (2015). The measurement of cores from each loin was recorded as the average of a minimum of eleven replicates.

### 6.2.5 Sensory analysis

For meat sensory analysis was used a trained sensory panel with ten members. For each of the six sessions, five or six frozen loin joints were randomly selected and maintained for 24 h at 2 ºC. After thawing, the LL muscle were isolated and cooked in an electric oven at 170 ºC, until

the internal temperature reached 70 ºC, which was monitored using an internal thermocouple (Thermometer, Omega RDX4SD, Manchester, USA). The LL muscle was then trimmed of external connective tissue, cut into 1 x 1 x 1 cm subsamples and maintained in heated dishes at 40 ºC, until tasting. Meat attributes evaluated were odour, flavour, tenderness, juiciness, off-odour, off-flavour and overall acceptability. For sensory analysis a structured scale from 1 to 8 was applied, where: 1 – extremely soft, tough, dry, weak or unacceptable; and 8 – extremely intense, tender, juicy, strong or acceptable.

### 6.2.6 Statistical analysis

Data were analyzed as completely randomized design using the MIXED procedure of SAS (SAS Institute Inc., Cary NC). A single fixed factor was the diet and lamb was the experimental unit. The variance homogeneity was tested for a level of $P = 0.01$ and, when significant, the variance heterogeneity was accommodated in the model. Live weight was monitored five times during trial, and data were analyzed by random intercept regression model in order to estimate average daily gain (i.e. slopes). Individual daily DM intake (DMI) was averaged per week and a repeated measurements model was adjusted in order to estimate the average DMI per lamb during the experiment, expressed as g/day per lamb. Average daily gain (ADG), slaughter live weight, hot and cold carcass weights were adjusted to initial live weight. Dressing, carcass cuts and shoulder composition were adjusted to hot carcass weight, and FA composition of muscle and subcutaneous fat were adjusted to lipid content of muscle and subcutaneous fat, respectively. Data from meat sensory evaluation were analyzed considering observations from each panelist.

## 6. 3 Results

### 6.3.1 Intake and growth performance

The results of feed intake and growth performance of lambs are presented in Table 6.2. Dry matter intake was affected by diets ($P < 0.001$), with lower DMI in lambs fed CL2.5 diet (926 g/day) than in lambs from all other diets. Moreover, lambs fed Ex1.25 diet (1160 g/day) had lower DMI when compared to lambs from L diet (1319 g/day), while lambs fed C1.25 and Ex2.5 diets showed an intermediate DMI between Ex1.25 and L diets. As result of the lower DMI, with CL2.5 diet the intake of CP, NDF, ADF, ADL and ash was lower than with other diets ($P < 0.001$). Moreover, CP intake was lower with CL1.25 and Ex1.25 diets than with L

diet, and lambs from Ex2.5 diet showed an intermediate intake of CP. The intake of NDF, ADF, ADL and ash presented the same trend, and were lower with Ex1.25 diet than with L diet, while lambs fed Ex2.5 diet presented an intermediate intake. Supplemented diets with 2.5% of CT (CL2.5 and Ex2.5) reduced ether extract intake comparatively with L and CL1.25 diets, and lambs fed Ex1.25 diet had an intermediate intake. The highest intakes of total phenols and CT were observed in lambs fed supplemented diets with 2.5% of CT (22.6 g TAE/day and 27.6 g/day of total phenols and condensed tannins, respectively). Among diets supplemented with 1.25% of CT, utilization of *C. ladanifer* aerial part resulted in higher intake of total phenols and CT than *C. ladanifer* CT extract (17.2 *vs*. 13.8 g TAE/day for total phenols, and 20.6 *vs*. 15.0 g/day for CT in CL1.25 and Ex1.25 diets, respectively). Lambs fed L diet showed the lowest intakes of total phenols and CT (8.28 g TAE /day and 5.88 g/day, respectively). Lambs fed CL2.5 diet showed the lowest intake of total and individual FA ($P < 0.001$, 85.2 g/day of total FA). Lambs supplemented with both levels of *C. ladanifer* CT extract had lower intake of total and individual FA than lambs from L and CL1.25 diets (106.5 *vs*. 128 g/day of total FA), except for 18:3*n*-3 intake that was equal in Ex2.5 and L diets.

Average daily gain was affected by treatments ($P < 0.001$), with lowest values with CL2.5 diet (143 g/day). Diet Ex2.5 resulted in lower ADG than L diet, while supplemented diets with 1.25% of CT had an intermediate ADG between Ex2.5 and L diets. Slaughter live weight was lower with CL2.5 diet than with L, CL1.25 and Ex1.25 diets, and lambs from Ex2.5 diet had an intermediate slaughter live weight ($P = 0.033$). Lambs fed the CL2.5 diet presented higher feed conversion ratio than lambs from L, CL1.25 and Ex1.25 diets, while lambs fed the Ex2.5 diet showed an intermediate feed conversion ratio ($P = 0.019$).

**Table 6.2.** Effect of *Cistus ladanifer* (CL) and their CT extract (Ex) on nutrient intake and growth performance of lambs

| | Diets | | | | | *P*-value |
|---|---|---|---|---|---|---|
| | L | CL1.25 | CL2.5 | Ex1.25 | Ex2.5 | |
| Intake (g/day) | | | | | | |
| DM | 1319 ± 38.2$^{a}$ | 1234 ± 54.0$^{ab}$ | 926 ± 54.0$^{c}$ | 1160 ± 54.0$^{b}$ | 1262 ± 54.0$^{ab}$ | <0.001 |
| CP | 233 ± 6.4$^{a}$ | 199 ± 9.0$^{b}$ | 134 ± 9.0$^{c}$ | 195 ± 9.0$^{b}$ | 214 ± 9.0$^{ab}$ | <0.001 |
| Ether extract | 111 ± 3.3$^{a}$ | 112 ± 4.7$^{a}$ | 99.3 ± 4.72$^{b}$ | 101 ± 4.7$^{ab}$ | 99.7 ± 4.72$^{b}$ | 0.041 |
| NDF | 643 ± 18.2$^{a}$ | 595 ± 25.7$^{ab}$ | 415 ± 25.7$^{c}$ | 557 ± 25.7$^{b}$ | 599 ± 25.7$^{ab}$ | <0.001 |
| ADF | 469 ± 13.3$^{a}$ | 435 ± 18.8$^{ab}$ | 304 ± 18.8$^{c}$ | 412 ± 18.8$^{b}$ | 435 ± 18.8$^{ab}$ | <0.001 |
| ADL | 89.2 ± 2.62$^{a}$ | 86.3 ± 3.71$^{ab}$ | 62.6 ± 3.71$^{c}$ | 79.8 ± 3.71$^{b}$ | 88.4 ± 3.71$^{ab}$ | <0.001 |
| Ash | 162 ± 4.6$^{a}$ | 148 ± 6.6$^{ab}$ | 105 ± 6.6$^{c}$ | 146 ± 6.6$^{b}$ | 155 ± 6.6$^{ab}$ | <0.001 |
| Total phenols[1] | 8.28 ± 0.567$^{d}$ | 17.2 ± 0.80$^{b}$ | 23.3 ± 0.80$^{a}$ | 13.8 ± 0.80$^{c}$ | 21.9 ± 0.80$^{a}$ | <0.001 |
| Condensed tannins[2] | 5.88 ± 0.668$^{d}$ | 20.6 ± 0.95$^{b}$ | 26.9 ± 0.95$^{a}$ | 15.0 ± 0.95$^{c}$ | 28.1 ± 0.95$^{a}$ | <0.001 |
| Total FA | 127 ± 3.6$^{a}$ | 129 ± 5.1$^{a}$ | 85.2 ± 5.06$^{c}$ | 103 ± 5.1$^{b}$ | 110 ± 5.1$^{b}$ | <0.001 |
| 16:0 | 16.9 ± 0.472$^{a}$ | 17.0 ± 0.67$^{a}$ | 10.6 ± 0.67$^{c}$ | 13.5 ± 0.67$^{b}$ | 15.1 ± 0.67$^{b}$ | <0.001 |
| 18:0 | 5.06 ± 0.144$^{a}$ | 5.25 ± 0.203$^{a}$ | 3.46 ± 0.203$^{c}$ | 4.10 ± 0.203$^{b}$ | 4.45 ± 0.203$^{b}$ | <0.001 |
| *c*9-18:1 | 27.3 ± 0.76$^{a}$ | 27.3 ± 1.08$^{a}$ | 18.1 ± 1.08$^{c}$ | 22.1 ± 1.08$^{b}$ | 23.1 ± 1.08$^{b}$ | <0.001 |
| 18:2*n*-6 | 64.3 ± 1.79$^{a}$ | 63.9 ± 2.53$^{a}$ | 42.4 ± 2.53$^{c}$ | 52.0 ± 2.53$^{b}$ | 54.7 ± 2.53$^{b}$ | <0.001 |
| 18:3*n*-3 | 9.45 ± 0.272$^{ab}$ | 9.93 ± 0.384$^{a}$ | 6.34 ± 0.384$^{d}$ | 7.74 ± 0.384$^{c}$ | 8.71 ± 0.384$^{bc}$ | <0.001 |
| Initial live weight (kg) | 21.3 | 19.4 | 18.7 | 19.6 | 20.3 | |
| Slaughter live weight (kg)[3] | 29.4 ± 0.64$^{a}$ | 29.4 ± 0.84$^{a}$ | 26.0 ± 0.89$^{b}$ | 29.4 ± 0.84$^{a}$ | 28.1 ± 0.83$^{ab}$ | 0.033 |
| Av daily gain (g/d) [3] | 290 ± 14.4$^{a}$ | 254 ± 20.0$^{ab}$ | 143 ± 20.0$^{c}$ | 256 ± 20.0$^{ab}$ | 240 ± 20.0$^{b}$ | <0.001 |
| Feed conversion ratio[3, 4] | 5.34 ± 0.298$^{b}$ | 5.29 ± 0.391$^{b}$ | 6.77 ± 0.410$^{a}$ | 5.03 ± 0.387$^{b}$ | 6.13 ± 0.384$^{ab}$ | 0.019 |

[1] Tannic acid equivalents, [2] Condensed tannins quantified using purified *Cistus ladanifer* CT as standard, [3] Adjusted for initial live weight; [4] kg dry matter intake/kg weight increase. Values are means ± standard error of the mean. Means within a row with different letters are significantly different ($P < 0.05$)

### 6.3.2 Carcass and meat quality traits

The carcass and meat quality traits are presented in Table 6.3. The CL2.5 diet led to lower hot and cold carcass weights than L, CL1.25 and Ex1.25 diets, and Ex2.5 diet did not differ from other diets ($P < 0.05$). The proportion of high price joints were higher with CL2.5, Ex1.25 and Ex2.5 diets than with L diet, and CL1.25 diet showed similar proportion to that obtained in other diets ($P = 0.023$). Dressing and KKCF percentage were not affected by treatments, averaged 43.0% and 2.20%, respectively. Dissection of shoulder cut showed that diets had no effect on the percentage of muscle ($P = 0.904$), subcutaneous ($P = 0.743$) and intermuscular fat ($P = 0.451$), intermuscular/subcutaneous fat ratio ($P = 0.326$) and dissectible fat ($P = 0.623$), averaging 60.0, 7.70, 10.3, 1.46 and 18.0%, respectively. Lambs fed CL2.5 diet presented lower muscle/bone ratio than lambs fed L, CL1.25 and Ex1.25 diets (2.59 vs. 2.88%), and CL1.25 diet showed similar ratio to that obtained in other diets ($P = 0.043$). Carcasses from lambs fed the CL2.5 diet tended ($P = 0.055$) to present a higher bone percentage than lambs fed L, CL1.25 and Ex1.25 diets (23.3 *vs*. 20.9%).

Meat colour parameters, except coordinate *a**, were not influenced by diets, averaging 44.0 for *L**, 5.89 for *b**, 18.3 for *H** and 18.8 for C*. Redness (*a**) tended ($P = 0.054$) to be lower in meat from lambs fed supplemented diets with both levels of C. *ladanifer* CT extracts than in meat from lambs fed CL1.25 diet. Moreover, meat from lambs fed Ex2.5 diet had higher *a** values than those from L diet.

Meat pH averaged 5.74 and did not differ among treatments ($P = 0.376$). Shear force was higher in meat from lambs fed *C. ladanifer* CT extract diets (Ex1.25 and Ex2.5) than in those lambs fed other diets ($P < 0.001$; 28.0 *vs*. 24.5 N). Meat from lambs fed Ex1.25 diet had higher cooking losses than those from lambs fed L and CL2.5 diets, while for CL1.25 and Ex2.5 diets was found similar cooking losses to that obtained in other diets ($P = 0.016$). Sensory attributes of meat were not affected by treatments ($P > 0.05$). Meat was considered as tender (7.2), with moderate juiciness (5.9), mild odour (3.0), mild flavor (2.9) and good acceptability (6.5). The intensity of off-odour and off-flavour was less than 1.

**Table 6.3.** Effect of *Cistus ladanifer* (CL) and their CT extract (Ex) on carcass traits and meat quality of lambs

| | Diets | | | | | *P*-value |
|---|---|---|---|---|---|---|
| | L | CL1.25 | CL2.5 | Ex1.25 | Ex2.5 | |
| *Carcass traits* | | | | | | |
| Hot carcass weight (kg)[1] | 12.8 ± 0.30[a] | 12.4 ± 0.39[a] | 11.2 ± 0.41[b] | 12.7 ± 0.39[a] | 12.2 ± 0.39[ab] | 0.041 |
| Cold carcass weight (kg) [1] | 12.3 ± 0.29[a] | 11.8 ± 0.38[a] | 10.4 ± 0.40[b] | 12.0 ± 0.38[a] | 11.4 ± 0.37[ab] | 0.017 |
| Dressing percentage (%)[1] | 43.7 ± 0.61 | 42.2 ± 0.86 | 42.9 ± 0.86 | 43.1 ± 0.86 | 43.2 ± 0.86 | 0.713 |
| KKCF (%)[2] | 2.60 ± 0.22 | 2.47 ± 0.27 | 1.83 ± 0.32 | 2.27 ± 0.27 | 1.81 ± 0.27 | 0.171 |
| High price joints (%)[2] | 50.9 ± 0.40[b] | 52.1 ± 0.49[ab] | 52.9 ± 0.59[a] | 52.5 ± 0.49[a] | 53.0 ± 0.49[a] | 0.023 |
| Shoulder tissues (%)[2] | | | | | | |
| Muscle | 59.5 ± 0.65 | 60.1 ± 0.80 | 59.8 ± 0.95 | 60.3 ± 0.80 | 60.3 ± 0.80 | 0.904 |
| Bone | 20.8 ± 0.48 | 20.5 ± 0.60 | 23.3 ± 0.71 | 21.4 ± 0.59 | 21.7 ± 0.59 | 0.055 |
| Muscle/bone ratio | 2.87 ± 0.06[a] | 2.93 ± 0.07[a] | 2.59 ± 0.08[b] | 2.83 ± 0.07[a] | 2.79 ± 0.07[ab] | 0.043 |
| Subcutaneous fat | 8.26 ± 0.60 | 7.79 ± 0.74 | 6.65 ± 0.88 | 7.84 ± 0.73 | 7.97 ± 0.73 | 0.743 |
| Intermuscular fat | 10.9 ± 0.569 | 11.2 ± 0.707 | 9.94 ± 0.840 | 10.0 ± 0.701 | 9.60 ± 0.701 | 0.451 |
| Interm./subcutaneous fat ratio | 1.39 ± 0.13 | 1.49 ± 0.16 | 1.79 ± 0.19 | 1.33 ± 0.16 | 1.30 ± 0.16 | 0.326 |
| Dissectible fat | 19.1 ± 0.97 | 19.0 ± 1.20 | 16.6 ± 1.42 | 17.8 ± 1.19 | 17.6 ± 1.19 | 0.623 |
| *Meat quality traits* | | | | | | |
| pH | 5.73 ± 0.197 | 5.70 ± 0.028 | 5.74 ± 0.028 | 5.78 ± 0.028 | 5.74 ± 0.028 | 0.376 |
| Cooking losses (%) | 25.2 ± 0.75[b] | 26.5 ± 1.06[ab] | 23.8 ± 1.06[b] | 29.1 ± 1.06[a] | 26.5 ± 1.06[ab] | 0.016 |
| Shear force (N) | 25.7 ± 0.41[b] | 25.2 ± 0.57[b] | 25.3 ± 0.57[b] | 28.5 ± 0.60[a] | 27.5 ± 0.56[a] | <0.001 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Colour parameters | | | | | | |
| *L** | 42.8 ± 0.77 | 43.1 ± 1.09 | 45.7 ± 1.09 | 42.9 ± 1.09 | 45.3 ± 1.09 | 0.118 |
| *a** | 18.4 ± 0.41$^{ab}$ | 19.2 ± 0.57$^{a}$ | 17.5 ± 0.57$^{abc}$ | 17.3 ± 0.57$^{bc}$ | 16.3 ± 0.57$^{c}$ | 0.054 |
| *b** | 6.09 ± 0.26 | 5.94 ± 0.38 | 6.22 ± 0.38 | 5.39 ± 0.38 | 5.82 ± 0.38 | 0.544 |
| Chroma | 19.4 ± 0.42 | 20.1 ± 0.60 | 18.6 ± 0.60 | 18.2 ± 0.60 | 17.9 ± 0.60 | 0.078 |
| Hue-Angle | 18.3 ± 0.75 | 17.2 ± 1.06 | 19.6 ± 1.06 | 17.2 ± 1.06 | 19.1 ± 1.06 | 0.382 |
| Sensorial evaluation | | | | | | |
| Tenderness | 7.78 ± 0.396 | 6.96 ± 0.632 | 7.36 ± 0.557 | 6.77 ± 0.585 | 7.01 ± 0.648 | 0.598 |
| Juiciness | 6.05 ± 0.130 | 5.68 ± 0.208 | 6.02 ± 0.184 | 5.64 ± 0.193 | 6.04 ± 0.213 | 0.295 |
| Odour intensity | 2.95 ± 0.136 | 2.98 ± 0.218 | 3.03 ± 0.192 | 2.68 ± 0.202 | 3.24 ± 0.223 | 0.471 |
| Off-odour intensity | 0.70 ± 0.118 | 0.39 ± 0.189 | 0.47 ± 0.167 | 0.68 ± 0.175 | 0.56 ± 0.194 | 0.600 |
| Flavour intensity | 2.94 ± 0.182 | 3.27 ± 0.290 | 2.78 ± 0.256 | 2.56 ± 0.269 | 2.73 ± 0.297 | 0.437 |
| Off-flavour intensity | 0.91 ± 0.118 | 0.68 ± 0.189 | 0.51 ± 0.167 | 0.49 ± 0.175 | 0.80 ± 0.194 | 0.200 |
| Overall acceptability | 6.43 ± 0.107 | 6.25 ± 0.170 | 6.63 ± 0.150 | 6.45 ± 0.157 | 6.84 ± 0.174 | 0.129 |

[1] Adjusted for initial live weight; [2] Adjusted for hot carcass weight.

Dressing – (hot carcass weight / slaughter weight) * 100.

High price joints – leg + chump + loin + ribs.

KKCF – kidney knob channel fat.

Values are means ± standard error of the mean. Means within a row with different letters are significantly different ($P < 0.05$)

### 6.3.3 Fatty acid composition of meat

Lipid content and general FA composition of *Longissimus thoracis* muscle are presented in Table 6.4. Intramuscular fat and total FA content of meat did not differ ($P > 0.05$) among treatments, averaging 14.9 and 10.0 g/kg in fresh meat, respectively. The major FA was *c*9-18:1, representing between 243 and 314 mg/g of total FA. The content of *c*9-18:1 was affected by diets ($P < 0.001$), which was higher for L diet than for CL2.5, Ex1.25 and Ex2.5 diets, but similar to that observed in CL1.25 diet. Moreover, CL2.5 diet had similar levels of *c*9-18:1 to Ex2.5 diet and lower than in other diets. The second most abundant FA was 16:0, which was not affected by treatments (232 mg/g of total FA). This was followed by 18:0, which ranged from 167 to 197 m/g of total FA, and was affected by dietary treatment ($P = 0.004$). Meat from lambs fed CL2.5 diet had higher content of 18:0 than in those from lambs fed L, CL1.25 and Ex1.25 diets, but similar to that obtained with Ex2.5 diet. Total linear chain SFA did not differ among treatments ($P = 0.750$) averaging 452 mg/g total FA. Beyond the 18:0, only 12:0 and 20:0 were affected by dietary treatments among linear chain SFA. Inclusion of 250 g/kg of *C. ladanifer* in diet (CL2.5) resulted in higher 20:0 content in meat than other diets, and higher amounts of 12:0 than with L, CL1.25 and Ex2.5 diets.

Total BCFA content averaged 8.99 mg/g of total FA, and neither the sum neither the individual BCFA were affected by treatments ($P > 0.05$). In addition to *c*9-18:1, the *c*9-16:1 was one of the *cis*-MUFA affected by dietary treatments, and its content was higher in meat from lambs fed L and CL1.25 diets than in those from lambs fed other diets ($P = 0{,}002$; 8.39 *vs*. 6.46 mg/g of total FA). The L diet showed similar sum of *cis*-MUFA to that obtained with CL1.25 diet, and higher that CL2.5, Ex1.25 and Ex2.5 diets ($P < 0.001$). Independently of the way of CT supply to diets, the sum of *cis*-MUFA was similar for each dose of CT supplementation. Total PUFA content and all individual *n*6- and *n*3-PUFA did not differ among treatments. Total PUFA averaging 110 mg/g total FA, with 18:2 *n*-6 as major PUFA in intramuscular fat (76.5 mg/g total FA).

**Table 6.4.** Effect of *Cistus ladanifer* (CL) and their CT extract (Ex) on intramuscular fat and fatty acid (FA) composition (mg/g of total fatty acid) of LT of lambs

| | Diets | | | | | *P*-value |
|---|---|---|---|---|---|---|
| | L | CL1.25 | CL2.5 | Ex1.25 | Ex2.5 | |
| Intramuscular fat (g/kg meat) | 16.5 ± 1.44 | 17.3 ± 1.62 | 13.3 ± 1.62 | 14.2 ± 1.62 | 13.4 ± 1.62 | 0.232 |
| Total FA (g/kg meat) [1] | 9.93 ± 0.702 | 9.95 ± 0.999 | 8.63 ± 0.994 | 12.00 ± 0.979 | 9.59 ± 0.992 | 0.201 |
| FA profile[1] | | | | | | |
| LC-SFA[2] | | | | | | |
| 10:0 | 1.15 ± 0.120 | 1.11 ± 0.171 | 1.57 ± 0.171 | 1.31 ± 0.168 | 1.30 ± 0.170 | 0.348 |
| 12:0 | 2.94 ± 0.465$^{b}$ | 3.04 ± 0.662$^{b}$ | 5.69 ± 0.659$^{a}$ | 4.21 ± 0.649$^{ab}$ | 3.36 ± 0.657$^{b}$ | 0.025 |
| 14:0 | 28.0 ± 2.50 | 29.6 ± 3.56 | 36.4 ± 3.754 | 33.6 ± 3.49 | 28.2 ± 3.53 | 0.317 |
| 15:0 | 4.55 ± 0.414 | 5.34 ± 0.590 | 5.81 ± 0.587 | 4.90 ± 0.578 | 4.61 ± 0.585 | 0.431 |
| 16:0 | 239 ± 6.4 | 237 ± 9.7 | 219 ± 9.9 | 230 ± 8.9 | 237 ± 9.0 | 0.497 |
| 17:0 | 7.76 ± 0.315 | 8.40 ± 0.449 | 8.33 ± 0.446 | 7.81 ± 0.439 | 8.08 ± 0.4945 | 0.692 |
| 18:0 | 167 ± 4.1$^{c}$ | 178 ± 5.9$^{bc}$ | 197 ± 5.9$^{a}$ | 174 ± 5.8$^{bc}$ | 188 ± 5.8$^{ab}$ | 0.004 |
| 20:0 | 1.16 ± 0.082$^{d}$ | 1.77 ± 0.117$^{b}$ | 2.86 ± 0.116$^{a}$ | 1.31 ± 0.114$^{cd}$ | 1.50 ± 0.116$^{bc}$ | <0.001 |
| 21:0 | 0.44 ± 0.179 | 0.81 ± 0.255 | 0.39 ± 0.254 | 0.30 ± 0.250 | 0.73 ± 0.253 | 0.520 |
| Sum | 440 ± 9.2 | 452 ± 13.1 | 464 ± 13.0 | 445 ± 12.8 | 460 ± 13.0 | 0.750 |
| BCFA[3] | | | | | | |
| iso-15:0 | 0.82 ± 0.051 | 0.72 ± 0.073 | 0.75 ± 0.072 | 0.81 ± 0.071 | 0.069 ± 0.072 | 0.543 |
| anteiso-15:0 | 1.28 ± 0.126 | 1.23 ± 0.180 | 1.71 ± 0.179 | 1.43 ± 0.176 | 1.24 ± 0.178 | 0.290 |
| iso-16:0 | 1.28 ± 0.061 | 1.28 ± 0.086 | 1.33 ± 0.086 | 1.41 ± 0.085 | 1.27 ± 0.086 | 0.757 |
| iso-17:0 | 2.40 ± 0.157 | 2.42 ± 0.223 | 2.68 ± 0.222 | 2.63 ± 0.219 | 2.20 ± 0.221 | 0.519 |
| anteiso-17:0 | 2.57 ± 0.147 | 2.32 ± 0.209 | 2.30 ± 0.208 | 2.68 ± 0.205 | 2.08 ± 0.208 | 0.237 |
| iso-18:0 | 0.75 ± 0.036 | 0.69 ± 0.052 | 0.63 ± 0.052 | 0.70 ± 0.050 | 0.65 ± 0.052 | 0.392 |
| Sum | 9.10 ± 0.512 | 8.65 ± 0.728 | 9.40 ± 0.725 | 9.65 ± 0.714 | 8.13 ± 0.723 | 0.575 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *cis*-MUFA[4] | | | | | | |
| *c*9-14:1 | 0.63 ± 0.064 | 0.65 ± 0.090 | 0.62 ± 0.090 | 0.71 ± 0.089 | 0.50 ± 0.090 | 0.535 |
| *c*9-16:1 | 8.33 ± 0.346$^{a}$ | 8.45 ± 0.492$^{a}$ | 5.94 ± 0.490$^{b}$ | 7.03 ± 0.482$^{b}$ | 6.42 ± 0.488$^{b}$ | 0.002 |
| *c*9-17:1 | 3.04 ± 0.189 | 3.56 ± 0.269 | 2.77 ± 0.268 | 2.84 ± 0.264 | 2.76 ± 0.267 | 0.236 |
| *c*9-18:1 | 314 ± 7.9$^{a}$ | 296 ± 11.3$^{ab}$ | 243 ± 11.2$^{c}$ | 278 ± 11.1$^{b}$ | 273 ± 11.2$^{bc}$ | <0.001 |
| *c*11-18:1 | 9.28 ± 0.390 | 8.57 ± 0.555 | 8.65 ± 0.553 | 8.68 ± 0.544 | 8.96 ± 0.551 | 0.790 |
| Sum | 335 ± 8.2$^{a}$ | 318 ± 11.7$^{ab}$ | 261 ± 11.6$^{c}$ | 297 ± 11.4$^{b}$ | 292 ± 11.6$^{bc}$ | <0.001 |
| *n*6-PUFA | | | | | | |
| 18:2*n*-6 | 63.2 ± 7.91 | 75.0 ± 11.26 | 88.6 ± 11.21 | 74.8 ± 11.804 | 80.9 ± 11.18 | 0.458 |
| 20:2*n*-6 | 0.49 ± 0.039 | 0.44 ± 0.056 | 0.51 ± 0.056 | 0.49 ± 0.055 | 0.55 ± 0.055 | 0.719 |
| 20:3*n*-6 | 1.26 ± 0.243 | 1.17 ± 0.346 | 1.96 ± 0.344 | 1.60 ± 0.339 | 1.76 ± 0.343 | 0.445 |
| 20:4*n*-6 | 9.09 ± 2.666 | 9.80 ± 3.80 | 16.0 ± 3.78 | 12.5 ± 3.72 | 13.0 ± 3.77 | 0.659 |
| 22:4*n*-6 | 0.60 ± 0.159 | 0.52 ± 0.227 | 0.80 ± 0.226 | 0.78 ± 0.222 | 0.76 ± 0.225 | 0.881 |
| Sum | 74.6 ± 10.60 | 86.9 ± 15.08 | 108 ± 15.0 | 90.2 ± 14.78 | 96.9 ± 14.97 | 0.494 |
| *n*3-PUFA | | | | | | |
| 18:3*n*-3 | 8.09 ± 0.981 | 9.48 ± 1.396 | 10.8 ± 1.39 | 10.1 ± 1.37 | 10.7 ± 1.39 | 0.478 |
| 20:3*n*-3 | 0.20 ± 0.025 | 0.19 ± 0.035 | 0.26 ± 0.035 | 0.26 ± 0.035 | 0.24 ± 0.035 | 0. 442 |
| 20:5*n*-3 | 1.66 ± 0.578 | 1.71 ± 0.823 | 3.34 ± 0.819 | 2.43 ± 0.807 | 2.48 ± 0.817 | 0.558 |
| 22:5*n*-3 | 3.00 ± 0.844 | 2.97 ± 1.201 | 5.06 ± 1.195 | 4.00 ± 1.177 | 4.16 ± 1.192 | 0.682 |
| 22:6*n*-3 | 0.58 ± 0.210 | 0.74 ± 0.299 | 1.02 ± 0.298 | 1.05 ± 0.293 | 0.91 ± 0.297 | 0.679 |
| Sum | 13.5 ± 2.54 | 15.1 ± 3.62 | 20.5 ± 3.60 | 17.8 ± 3.54 | 18.5 ± 3.59 | 0.586 |
| PUFA[5] | 89.8 ± 13.16 | 104 ± 18.7 | 131 ± 18.6 | 110 ± 18.4 | 117 ± 18.6 | 0.502 |

[1] Adjusted for total lipids content; [2] LC-SFA, linear chain fatty acids; [3] BCFA, branched chain fatty acids; [4] *cis*-MUFA, sum of *cis*-MUFA excluding the biohydrogenation intermediates; [5] PUFA, Sum of all-*cis*, methylene interrupted polyunsaturated fatty acids.

Values are means ± standard error of the mean. Means within a row with different letters are significantly different ($P < 0.05$)

The detailed profile of C18 BH intermediates (BI) in meat are presented in Table 6.5. The major BI was the *t*11-18:1, which was higher in lambs fed Ex1.25 diet than in lambs fed all other diets ($P$ = 0.006; 58.5 mg/g total FA). Moreover, higher *t*11-18:1 content was found in L diet comparatively to that obtained with CL1.25 diet (46.8 *vs.* 35.7 mg/g total FA), while CL2.5 and Ex2.5 diets showed intermediate amounts between L and CL1.25 diets. Feeding CL2.5 diet resulted in higher levels of *t*12-, *t*16- plus *c*14- and *c*12-18:1 in meat when compared with those from lamb fed L, Ex1.25 and Ex2.5 diets (8.51 *vs*. 6.50, 4.43 *vs*. 3.71 and 19.0 *vs*. 10.6 mg/g of total FA, respectively, for *t*12-, *t*16- plus *c*14- and *c*12-18:1), whereas CL1.25 presented an intermediate content ($P < 0.05$). The *t*15-18:1 was higher for CL2.5 diet than for other diets ($P$ = 0.002; 3.91 *vs*. 2.86 mg/g of total FA). Total of 18:1 BI content did not differ among treatments ($P$ = 0.272) and averaged in 102.8 mg/g of total FA. Total 18:2 BI content was not influenced by diets, averaged 23.6 mg/g of total FA. The *c*9,*t*11-18:2 was the predominant 18:2 BI, ranging from 12.0 mg/g of total FA in Ex1.25 diet to 7.82 mg/g of total FA in CL1.25 diet ($P$ = 0.044). The *c*12,*c*15-18:2 was higher for CL2.5 diet than for L, Ex1.25 and Ex2.5 diets, but to similar to than obtained for CL1.25 diet ($P$ = 0.004). Total BI content also did not differ among treatments ($P$ = 0.268), averaged 127 mg/g of total FA. Total *trans*-18:1 isomers content was not affected by treatments, whereas the sum of *cis*-18:1 isomers was higher with CL2.5 diet than that observed with L, Ex1.25 and Ex2.5 diets, and intermediate levels was found with CL1.25 diet.

**Table 6.5**. Effect of *Cistus ladanifer* (CL) and their CT extract (Ex) on C18 BH intermediates (mg/g of total fatty acid) present in LT of lambs

| | Diets | | | | | *P*-value |
|---|---|---|---|---|---|---|
| | L | CL1.25 | CL2.5 | Ex1.25 | Ex2.5 | |
| 18:1 isomers | | | | | | |
| *t*6/*t*7/*t*8- | 4.14 ± 0.220 | 3.89 ± 0.313 | 4.40 ± 0.311 | 4.68 ± 0.307 | 4.05 ± 0.311 | 0.411 |
| *t*9- | 3.89 ± 0.143 | 3.602 ± 0.203 | 3.94 ± 0.202 | 4.15 ± 0.199 | 3.62 ± 0.201 | 0.287 |
| *t*10- | 10.6 ± 1.12 | 11.2 ± 1.60 | 10.9 ± 1.59 | 8.90 ± 1.57 | 10.8 ± 1.58 | 0.849 |
| *t*11- | $46.8 \pm 2.84^{b}$ | $35.7 \pm 4.05^{c}$ | $42.5 \pm 4.03^{bc}$ | $58.5 \pm 3.97^{a}$ | $44.2 \pm 4.02^{bc}$ | 0.006 |
| *t*12- | $6.19 \pm 0.383^{b}$ | $7.02 \pm 0.546^{ab}$ | $8.51 \pm 0.544^{a}$ | $6.51 \pm 0.535^{b}$ | $6.81 \pm 0.542^{b}$ | 0.027 |
| *t*15- | $2.51 \pm 0.180^{b}$ | $3.13 \pm 0.257^{b}$ | $3.91 \pm 0.255^{a}$ | $2.74 \pm 0.252^{b}$ | $3.04 \pm 0.255^{b}$ | 0.002 |
| *t*16-[1] | $3.65 \pm 0.140^{b}$ | $3.92 \pm 0.199^{ab}$ | $4.43 \pm 0.198^{a}$ | $3.76 \pm 0.195^{b}$ | $3.73 \pm 0.198^{b}$ | 0.004 |
| *c*12- | $10.3 \pm 1.26^{b}$ | $14.0 \pm 1.79^{ab}$ | $19.0 \pm 1.78^{a}$ | $9.91 \pm 1.753^{b}$ | $11.6 \pm 1.77^{b}$ | 0.004 |
| *c*13- | 0.83 ± 0.080 | 1.00 ± 0.113 | 0.81 ± 0.113 | 0.73 ± 0.111 | 0.84 ± 0.112 | 0.562 |
| *c*15- | 0.80 ± 0.056 | 0.99 ± 0.080 | 1.04 ± 0.079 | 0.85 ± 0.078 | 0.92 ± 0.079 | 0.112 |
| *c*16- | 1.16 ± 0.068 | 1.21 ± 0.097 | 1.44 ± 0.097 | 1.05 ± 0.095 | 1.14 ± 0.096 | 0.070 |
| Sum | 100 ± 4.2 | 94.3 ± 5.97 | 110 ± 5.94 | 110 ± 5.85 | 99.7 ± 5.92 | 0.272 |
| 18:2 isomers | | | | | | |
| *t*11,*c*15- | 1.72 ± 0.091 | 1.69 ± 0.129 | 1.68 ± 0.128 | 1.84 ± 0.126 | 1.69 ± 0.128 | 0.886 |
| *c*9,*c*15- | 0.35 ± 0.029 | 0.39 ± 0.041 | 0.38 ± 0.041 | 0.37 ± 0.040 | 0.35 ± 0.041 | 0.867 |
| *c*12,*c*15- | $0.20 \pm 0.046^{c}$ | $0.37 \pm 0.065^{ab}$ | $0.55 \pm 0.065^{a}$ | $0.31 \pm 0.064^{bc}$ | $0.33 \pm 0.065^{bc}$ | 0.004 |
| *c*9,*t*11- | $11.3 \pm 0.88^{ab}$ | $7.82 \pm 1.254^{c}$ | $8.32 \pm 1.247^{bc}$ | $12.0 \pm 1.23^{a}$ | $8.69 \pm 1.244^{abc}$ | 0.044 |
| Sum | 25.8 ± 1.06 | 22.6 ± 1.51 | 22.1 ± 1.50 | 25.6 ± 1.48 | 21.9 ± 1.49 | 0.098 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 18:3 isomers | | | | | | |
| *c*9,*t*11,*c*15[2] | 1.68 ± 0.267 | 1.57 ± 0.380 | 2.41 ± 0.378 | 2.23 ± 0.373 | 1.79 ± 0.377 | 0.441 |
| BH intermediates (BI)[3] | 126 ± 4.61 | 117 ± 6.56 | 132 ± 6.53 | 136 ± 6.43 | 122 ± 6.51 | 0.268 |
| *trans*-BI sum[4] | 77.7 ± 3.49 | 68.5 ± 4.97 | 78.6 ± 4.95 | 89.3 ± 4.87 | 76.3 ± 4.94 | 0.087 |
| *cis*-BI sum[5] | 21.6 ± 1.61$^{b}$ | 24.8 ± 2.30$^{ab}$ | 29.9 ± 2.29$^{a}$ | 20.4 ± 2.25$^{b}$ | 22.5 ± 2.28$^{b}$ | 0.036 |

All C18 biohydrogenation intermediates were adjusted for total lipids content

[1] includes small amounts of *c*14-18:1; [2] includes the 20:3*n*-9; [3] BI, sum of biohydrogenation intermediates, i.e. all fatty acids listed in the table; [4] *trans*-BI sum, sum of all *trans* fatty acids listed in the table; [5] *cis*-BI sum, sum of all *cis* fatty acids listed in the table.

Values are means ± standard error of the mean. Means within a row with different letters are significantly different ($P < 0.05$)

### 6.3.4 Fatty acid composition of subcutaneous fat

Lipid content and general FA composition of subcutaneous fat are presented in Table 6.6. Lipid content of subcutaneous fat was affected by dietary treatments ($P$ = 0.003). Lipid content of subcutaneous fat was lower for CL2.5 diet compared with L, CL1.25 and Ex1.25 diets, but similar to that obtained for Ex2.5 diet.

Globally, FA composition of subcutaneous fat presented the same trend of meat. The major FA in subcutaneous fat was *c*9-18:1, and its content was higher ($P$ = 0.004) in lambs fed L diet than in lambs fed CL2.5 and Ex1.25 diets, with intermediate amount in other diets ($P$ = 0.004). This was followed by 16:0 and 18:0. For L, CL1.25 and Ex1.25 diets, 16:0 and 18:0 were the second and third largest FA in subcutaneous fat, but for CL2.5 and Ex2.5 diets the second major FA was 18:0, followed by the 16:0. The 16:0 content did not differ among dietary treatments (averaging 212.6 mg/g of total FA). The 18:0 content was affected by diets (P = 0.028), and was higher for CL2.5 and Ex2.5 comparatively with L diet (220 *vs*. 182 mg/g of total FA), while CL1.25 and Ex1.25 diets showed intermediate values. In addition to 18:0, only the 20:0 was affected by dietary treatments among linear chain SFA ($P$ < 0.001). The CL2.5 diet had the highest 20:0 content, followed by CL1.25 and Ex1.25 diets, and intermediate levels for Ex2.5 diet between CL1.25 and Ex1.25 diets. The L diet showed the lowest 20:0 content (3.5-fold lower than CL2.5 diet). Total linear chain SFA content was affected by dietary treatments ($P$ = 0.004), and its content was higher in subcutaneous fat from lambs fed CL2.5 diet than in lambs fed L diet (508 *vs*. 454 mg/g of total FA), and in other diets were found intermediate values.

Among BCFA, only anteiso-17:0 and iso-18:0 were affected by dietary treatments. The major BCFA was anteiso-17:0, which was higher for L diet than in other diets ($P$ = 0.010; 4.28 *vs*. 2.97 mg/g of total FA). Inclusion of 25% of *C. ladanifer* aerial part in diet (CL2.5) led to a lower iso-18:0 content than in L, Ex1.25 and Ex2.5 diets (0.50 *vs*. 0.89 mg/g of total FA), and for CL1.25 diet was observed an intermediate value. The total BCFA content showed a tendency ($P$ = 0.061) to be higher in subcutaneous fat from lambs fed L diet than in those from lambs fed CL1.25, CL2.5 and Ex2.5 diets (11.4 *vs*. 9.31 mg/g of total FA).

The total *cis*-MUFA content was lower with CL2.5 diet than with L and CL1.25 diets, and lower in lambs fed with both diets supplemented with *C. ladanifer* CT extracts (Ex1.25 and Ex2.5) when compared with L diet ($P$ = 0.004). Beyond *c*9-18:1, none of other individual *cis*-MUFA was affected by dietary treatments. However, subcutaneous fat from lambs fed L diet tended to have higher content of *c*9-16:1 ($P$ = 0.080) than those from lambs fed CL2.5, Ex1.25 and Ex2.5 diets, and higher *c*11-18:1 ($P$ = 0.059) content than in lambs fed Ex2.5 diet.

The total PUFA content and all individual *n*6-PUFA were not affected by treatments ($P > 0.05$), and averaged 53.5 mg/g total FA and 40.1 mg/g total FA, respectively. The 18:3*n*-3 was affected by dietary treatments ($P = 0.011$), and was higher for CL1.25, Ex1.25 and Ex2.5 diets than for L diet (10.1 *vs*. 8.37 mg/g of total FA), while in CL2.5 diets were found intermediate 18:3*n*-3 content. The content of 20:5*n*-3 was higher in subcutaneous fat in lambs fed CL2.5, Ex1.25 and Ex2.5 diets than in those from lambs fed L diet (0.27 *vs*. 0.15 mg/g of total FA), and lambs fed CL1.25 diets had intermediate values ($P = 0.021$). The total of *n*3-PUFA was also affected by treatments ($P = 0.011$), which presented the same trend as 18:3*n*-3.

The detailed profile of C18 BI in subcutaneous fat are presented in Table 6.7. The major BI was the *t*11-18:1 that was higher in lambs fed Ex1.25 diet than in lambs fed other diets ($P = 0.001$; 96.7 mg/g of total FA). Moreover, higher *t*11-18:1 content was found in L diet comparatively to CL1.25 diet (72.6 *vs*. 54.1 mg/g of total FA), while CL2.5 and Ex2.5 diets showed intermediate amounts between L and CL1.25 diets. The *t*12, *t*16 and *c*12-18:1 were affected by diets ($P < 0.05$), with higher contents for CL2.5 diets than to for L diet. Total of 18:1 BI content was higher with Ex1.25 diets ($P = 0.014$) than L, CL1.25 and Ex2.5 diets (170 *vs*. 143 mg/g of total FA), while CL2.5 diet led to intermediate values.

The *c*9,*t*11-18:2 was the predominant 18:2 BI in subcutaneous fat, with higher content in lambs fed Ex1.25 and L diets than in other treatments ($P < 0.001$; 17.2 *vs*. 12.6 mg/g of total FA). Inclusion of *C. ladanifer* aerial part in diets (both CL1.25 and CL2.5) resulted in higher content of *c*12,*c*15-18:2 comparatively with fed L diet ($P = 0.035$; 0.55 *vs*. 0.29 mg/g of total FA), while intermediate contents were found with *C. ladanifer* CT extracts supplemented diets (Ex1.25 and Ex2.5). Sum of 18:2 BI was higher for L diet than for CL1.25, CL2.5 and Ex2.5 diets ($P = 0.023$; 33.1 *vs*. 28.6 mg/g of total FA), and with Ex1.25 diet was obtained intermediate values.

Total BI and total *trans*-18:1 isomers contents were affected by dietary treatments ($P < 0.05$), and were higher for Ex1.25 diet than for L, CL1.25 and Ex2.5 diets (201 *vs*. 173 and 149 vs. 121 mg/g of total FA, respectively, for total BI and total *trans*-18:1), with intermediate contents in the CL2.5 diet. Total *cis*-18:1 isomers content tended ($P = 0.088$) to be higher in lambs fed CL2.5 diet than fed L diet.

**Table 6.6.** Effect of *Cistus ladanifer* (CL) and their CT extract (Ex) on subcutaneous fat lipids and fatty acid (FA) composition (mg/g of total fatty acid) of subcutaneous fat of lambs

| | Diets | | | | | *P*-value |
|---|---|---|---|---|---|---|
| | L | CL1.25 | CL2.5 | Ex1.25 | Ex2.5 | |
| Lipids (mg/g fresh subc. fat) | 567 ± 32.3$^{ab}$ | 624 ± 45.6$^{a}$ | 362 ± 45.6$^{c}$ | 536 ± 45.6$^{ab}$ | 470 ± 45.6$^{bc}$ | 0.003 |
| FA profile[1] | | | | | | |
| LC-SFA[2] | | | | | | |
| 10:0 | 1.55 ± 0.127 | 1.52 ± 0.187 | 2.04 ± 0.205 | 1.55 ± 0.174 | 1.60 ± 0.177 | 0.364 |
| 12:0 | 2.52 ± 0.431 | 2.77 ± 0.638 | 5.07 ± 0.697 | 3.03 ± 0.592 | 2.52 ± 0.603 | 0.053 |
| 14:0 | 31.8 ± 2.64 | 33.8 ± 3.91 | 42.8 ± 4.27 | 34.5 ± 3.63 | 32.4 ± 3.69 | 0.307 |
| 15:0 | 6.29 ± 0.340 | 6.44 ± 0.503 | 6.88 ± 0.549 | 6.17 ± 0.467 | 6.27 ± 0.475 | 0.872 |
| 16:0 | 214 ± 4.3 | 214 ± 6.4 | 211 ± 6.9 | 215 ± 5.9 | 209 ± 6.0 | 0.949 |
| 17:0 | 14.0 ± 0.80 | 13.6 ± 1.18 | 10.6 ± 1.29 | 11.3 ± 1.09 | 13.4 ± 1.11 | 0.140 |
| 18:0 | 182 ± 6.9$^{b}$ | 199 ± 10.2$^{ab}$ | 223 ± 11.1$^{a}$ | 200 ± 9.5$^{ab}$ | 216 ± 9.6$^{a}$ | 0.028 |
| 20:0 | 1.54 ± 0.168$^{d}$ | 3.12 ± 0.249$^{b}$ | 5.32 ± 0.272$^{a}$ | 2.11 ± 0.232$^{c}$ | 2.78 ± 0.236$^{bc}$ | <0.001 |
| 21:0 | 0.23 ± 0.043 | 0.17 ± 0.063 | 0.30 ± 0.069 | 0.22 ± 0.059 | 0.21 ± 0.060 | 0.758 |
| Sum | 454 ± 6.6$^{b}$ | 476 ± 9.8$^{ab}$ | 508 ± 10.7$^{a}$ | 474 ± 9.1$^{ab}$ | 484 ± 9.3$^{ab}$ | 0.004 |
| BCFA[3] | | | | | | |
| iso-15:0 | 1.09 ± 0.073 | 0.87 ± 0.109 | 0.93 ± 0.119 | 1.12 ± 0.101 | 0.96 ± 0.103 | 0.286 |
| anteiso-15:0 | 1.71 ± 0.135 | 1.52 ±0.199 | 1.81 ± 0.218 | 1.64 ± 0.185 | 1.49 ± 0.188 | 0.731 |
| iso-16:0 | 1.60 ± 0.074 | 1.48 ± 0.105 | 1.49 ± 0.115 | 1.56 ± 0.097 | 1.52 ± 0.099 | 0.836 |
| iso-17:0 | 1.93 ± 0.077 | 1.72 ± 0.114 | 1.85 ± 0.125 | 1.74 ± 0.106 | 1.75 ± 0.108 | 0.440 |
| anteiso-17:0 | 4.28 ± 0.272$^{a}$ | 3.18 ± 0.403$^{b}$ | 2.40 ± 0.440$^{b}$ | 3.19 ± 0.374$^{b}$ | 3.10 ± 0.380$^{b}$ | 0.010 |
| iso-18:0 | 0.79 ± 0.037$^{a}$ | 0.67 ± 0.055$^{ab}$ | 0.50 ± 0.060$^{b}$ | 0.70 ± 0.051$^{a}$ | 0.68 ± 0.052$^{a}$ | 0.007 |
| Sum | 11.4 ± 0.50 | 9.45 ± 0.745 | 8.96 ± 0.814 | 9.95 ± 0.692 | 9.52 ± 0.704 | 0.061 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *cis*-MUFA[4] | | | | | | |
| *c*9-14:1 | 0.78 ± 0.079 | 0.73 ± 0.116 | 0.53 ± 0.127 | 0.64 ± 0.108 | 0.52 ± 0.11 | 0.382 |
| *c*9-16:1 | 8.78 ± 0.583 | 8.38 ± 0.862 | 6.08 ± 0.941 | 6.70 ± 0.800 | 6.33 ± 0.814 | 0.080 |
| *c*9-17:1 | 5.33 ± 0.600 | 5.11 ± 0.888 | 3.02 ± 0.970 | 3.10 ± 0.824 | 3.58 ± 0.839 | 0.155 |
| *c*9-18:1 | 294 ± 7.0$^{a}$ | 278 ± 10.4$^{ab}$ | 239 ± 11.4$^{c}$ | 253 ± 9.6$^{bc}$ | 270 ± 9.8$^{ab}$ | 0.004 |
| *c*11-18:1 | 7.22 ± 0.196 | 6.59 ± 0.291 | 6.12 ± 0.317 | 6.87 ± 0.270 | 6.54 ± 0.275 | 0.059 |
| Sum | 316 ± 7.9$^{a}$ | 299 ± 11.7$^{ab}$ | 256 ± 12.8$^{c}$ | 271 ± 10.9$^{bc}$ | 287 ± 11.1$^{bc}$ | 0.004 |
| *n*6-PUFA | | | | | | |
| 18:2*n*-6 | 37.0 ± 2.41 | 43.7 ± 3.56 | 37.3 ± 3.89 | 40.3 ± 3.30 | 42.0 ± 3.36 | 0.467 |
| 20:2*n*-6 | 0.28 ± 0.025 | 0.30 ± 0.037 | 0.26 ± 0.040 | 0.32 ± 0.034 | 0.32 ± 0.035 | 0.613 |
| 20:3*n*-6 | 0.33 ± 0.022 | 0.36 ± 0.033 | 0.29 ± 0.036 | 0.28 ± 0.031 | 0.34 ± 0.031 | 0.382 |
| 20:4*n*-6 | 1.05 ± 0.063 | 1.24 ± 0.094 | 1.15 ± 0.103 | 0.96 ± 0.087 | 1.17 ± 0.089 | 0.174 |
| 22:4*n*-6 | 0.11 ± 0.029 | 0.10 ± 0.042 | 0.03 ± 0.046 | 0.09 ± 0.039 | 0.11 ± 0.040 | 0.700 |
| Sum | 38.7 ± 2.46 | 45.7 ± 3.64 | 39.1 ± 3.98 | 41.9 ± 3.38 | 43.9 ± 3.44 | 0.458 |
| *n*3-PUFA | | | | | | |
| 18:3*n*-3 | 8.37 ± 0.379$^{b}$ | 10.26 ± 0.560$^{a}$ | 9.00 ± 0.612$^{ab}$ | 9.47 ± 0.520$^{a}$ | 10.53 ± 0.529$^{a}$ | 0.011 |
| 20:3*n*-3 | 0.16 ± 0.024 | 0.18 ± 0.035 | 0.17 ± 0.039 | 0.21 ± 0.033 | 0.21 ± 0.034 | 0.674 |
| 20:5*n*-3 | 0.15 ± 0.026$^{b}$ | 0.24 ± 0.038$^{ab}$ | 0.26 ± 0.042$^{a}$ | 0.26 ± 0.035$^{a}$ | 0.29 ± 0.036$^{a}$ | 0.021 |
| 22:5*n*-3 | 0.89 ± 0.062 | 0.91 ± 0.092 | 1.06 ± 0.100 | 0.85 ± 0.085 | 0.84 ± 0.087 | 0.480 |
| 22:6*n*-3 | 0.27 ± 0.038 | 0.39 ± 0.057 | 0.26 ± 0.062 | 0.26 ± 0.053 | 0.30 ± 0.054 | 0.409 |
| Sum | 9.84 ± 0.411$^{b}$ | 11.97 ± 0.608$^{a}$ | 10.75 ± 0.664$^{ab}$ | 11.03 ± 0.565$^{a}$ | 12.18 ± 0.575$^{a}$ | 0.011 |
| PUFA[5] | 49.1 ± 2.78 | 58.2 ± 4.12 | 50.3 ± 4.50 | 53.6 ± 3.82 | 56.5 ± 3.89 | 0.295 |

[1] Adjusted for total lipids content; [2] LC-SFA, linear chain fatty acids; [3] BCFA, branched chain fatty acids; [4] *cis*-MUFA, sum of *cis*-MUFA excluding the biohydrogenation intermediates; [5] PUFA, Sum of all-*cis*, methylene interrupted polyunsaturated fatty acids.

Values are means ± standard error of the mean. Means within a row with different letters are significantly different ($P < 0.05$)

**Table 6.7.** Effect of *Cistus ladanifer* (CL) and their CT extract (Ex) on C18 BH intermediates (mg/g of total fatty acids) present in subcutaneous fat of lambs

| | Diets | | | | | *P*-value |
|---|---|---|---|---|---|---|
| | L | CL1.25 | CL2.5 | Ex1.25 | Ex2.5 | |
| 18:1 isomers | | | | | | |
| *t*6/*t*7/*t*8- | 6.59± 0.309 | 6.30 ± 0.456 | 7.07 ± 0.499 | 7.89 ± 0.424 | 6.72 ± 0.431 | 0.106 |
| *t*9- | 6.09 ± 0.632 | 4.77 ± 0.934 | 4.87± 1.021 | 5.81 ± 0.868 | 5.12 ± 0.883 | 0.672 |
| *t*10- | 23.3 ± 2.69 | 27.1 ± 3.98 | 24.4 ± 4.34 | 17.9 ± 3.69 | 22.2 ± 3.76 | 0.514 |
| *t*11- | 72.6 ± 4.67$^{b}$ | 54.1 ± 6.91$^{c}$ | 70.9 ± 7.55$^{bc}$ | 96.7 ± 6.42$^{a}$ | 67.6 ± 6.53$^{bc}$ | 0.001 |
| *t*12- | 7.83 ± 0.574$^{b}$ | 10.27 ± 0.849$^{a}$ | 11.30 ± 0.927$^{a}$ | 10.04 ± 0.788$^{a}$ | 7.43 ± 0.802$^{ab}$ | 0.019 |
| *t*15- | 5.77 ± 0.355 | 5.83 ± 0.525 | 6.39 ±0.573 | 5.54 ± 0.487 | 6.27 ± 0.496 | 0.783 |
| *t*16-[1] | 4.47 ± 0.323$^{b}$ | 5.47 ± 0.477$^{ab}$ | 6.58 ± 0.521$^{a}$ | 5.17 ± 0.443$^{ab}$ | 5.89 ± 0.451$^{a}$ | 0.023 |
| *c*12- | 9.66 ± 1.148$^{c}$ | 14.13 ± 1.698$^{ab}$ | 16.19 ± 1.855$^{a}$ | 10.75 ± 1.577$^{bc}$ | 11.00 ± 1.605$^{bc}$ | 0.026 |
| *c*13- | 0.78 ± 0.041 | 0.78 ± 0.060 | 0.66 ± 0.066 | 0.68 ± 0.056 | 0.68 ± 0.057 | 0.386 |
| *c*15- | 1.30 ± 0.124 | 1.31 ± 0.183 | 1.50 ± 0.200 | 1.16 ± 0.386 | 1.34 ± 0.173 | 0.798 |
| *c*16- | 1.17 ± 0.071 | 1.35 ± 0.105 | 1.52 ± 0.114 | 1.19 ± 0.097 | 1.30 ± 0.099 | 0.109 |
| sum | 147 ± 4.6$^{b}$ | 138 ± 6.8$^{b}$ | 157 ± 7.5$^{ab}$ | 170 ± 6.3$^{a}$ | 144 ± 6.5$^{b}$ | 0.014 |
| 18:2 isomers | | | | | | |
| *t*11,*c*15- | 2.49 ± 0.122 | 2.60± 0.180 | 2.65 ± 0.197 | 2.84 ± 0.168 | 2.49 ± 0.171 | 0.506 |
| *c*9,*c*15- | 0.43 ± 0.045 | 0.54 ± 0.067 | 0.49 ± 0.073 | 0.36 ± 0.062 | 0.41 ± 0.063 | 0.295 |
| *c*12,*c*15- | 0.29 ± 0.057$^{b}$ | 0.53 ± 0.084$^{a}$ | 0.57 ± 0.092$^{a}$ | 0.33 ± 0.078$^{ab}$ | 0.35 ± 0.080$^{ab}$ | 0.035 |
| *c*9,*t*11- | 17.3 ± 0.78$^{a}$ | 12.3 ± 1.16$^{b}$ | 12.5 ± 1.27$^{b}$ | 17.0 ± 1.08$^{a}$ | 13.1 ± 1.10$^{b}$ | <0.001 |
| sum | 33.1 ± 0.93$^{a}$ | 29.3 ± 1.38$^{b}$ | 28.0 ± 1.51$^{b}$ | 31.6 ± 1.28$^{ab}$ | 28.8 ± 1.31$^{b}$ | 0.023 |
| 18:3 isomers | | | | | | |
| *c*9,*t*11,*c*15[2] | 0.55 ± 0.043 | 0.57 ± 0.064 | 0.51 ± 0.070 | 0.64 ± 0.060 | 0.46 ± 0.061 | 0.359 |

| | | | | | | |
|---|---|---|---|---|---|---|
| BH intermediates (BI)[3] | 183 ± 4.6$^{b}$ | 168± 6.8$^{b}$ | 185 ± 7.4$^{ab}$ | 201 ± 6.3$^{a}$ | 173 ± 6.4$^{b}$ | 0.008 |
| *trans*- BI sum[4] | 127 ± 4.4$^{b}$ | 114 ± 6.5$^{b}$ | 131 ± 7.1$^{ab}$ | 149 ± 6.0$^{a}$ | 123 ± 6.1$^{b}$ | 0.006 |
| *cis*- BI sum[5] | 18.7 ± 1.27 | 22.8 ± 1.88 | 24.5 ± 2.05 | 19.5 ± 1.74 | 19.5 ± 1.77 | 0.088 |

All C18 biohydrogenation intermediates were adjusted for total lipids content

[1] includes small amounts of *c*14-18:1; [2] includes the 20:3*n*-9; [3] BI, sum of biohydrogenation intermediates, i.e. all fatty acids listed in the table; [4] *trans*-BI sum, sum of all *trans* fatty acids listed in the table; [5] *cis*-BI sum, sum of all *cis* fatty acids listed in the table.

Values are means ± standard error of the mean. Means within a row with different letters are significantly different ($P < 0.05$)

## 6. 4. Discussion

### 6.4.1. Intake and growth performance

*Cistus ladanifer* is considered an unbalanced feed with poor nutritional value and with high levels of antinutritional factors, as CT (Dentinho et al., 2005; Guerreiro et al., 2016b), so it would be expected that the inclusion of high levels of C. *ladanifer* in diets could depress the feed intake and growth performance. In fact, inclusion of 125 or 250 g/kg of *C. ladanifer* aerial part in diet affected its composition, particularly the crude protein content that reduced progressively with increasing levels of *C. ladanifer* in diets (162, 147 and 133 g/kg DM in L, CL1.25 and CL2.5 diets, respectively). However, in previous trials with inclusion of *C. ladanifer* aerial part in diets, no adverse effects on feed intake and lamb growth were observed (Francisco *et al.*, 2017; Francisco *et al.*, 2015; Jerónimo *et al.*, 2010), even with high levels of incorporation (250 g/kg of *C. ladanifer*) (Jerónimo *et al*., 2010). Contrary to previous results, in present work the incorporation of 250 g/kg of *C. ladanifer* in diet impaired the DMI and the growth performance of lambs. Such result may be due to higher levels of CT in CL2.5 diet than in diet used previously (26.6 *vs.* 20.7 g/kg DM of CT, respectively) for the same amount of *C. ladanifer* incorporation in diets (250 g CL/kg of DM, Jerónimo *et al*., (2010)). The CT are known by their possible negative effects on animal intake, protein digestibility and animal performances (Makkar, 2003a). Condensed tannins are able to form indigestible complexes with several molecules, as proteins, polysaccharides or minerals, decreasing nutrient digestibility and feed intake (Makkar, 2003a). Additionally, the low intake of CT-rich feeds is attributed to a low palatability, due to the sensation of astringency that CT confer by binding with salivary proteins, which hamper the saliva from exercising its lubricant role in the mouth, giving an unpleasant feeling of dryness and harshness (Lesschaeve & Noble, 2005).

The depressive effect of dietary CT on growth performance was also found in diet supplemented with highest level of *C. ladanifer* CT extract (Ex2.5), although less pronounced than in CL2.5 diet. The Ex2.5 diet had minor effect on DMI, observing similar DMI between lambs fed Ex1.25 diet and unsupplemented lambs. Although, both diets with highest levels of CT (CL2.5 and Ex2.5) has been formulated to achieve the same CT levels, CL2.5 diet showed higher CT concentration (26.6 g/kg DM of CT) than Ex2.5 diet (20.3 g/kg DM of CT), which associated to nutrient dilution due to high levels of *C. ladanifer* in diet, may explain the more pronounced effects of CL2.5 diet than Ex2.5 diet on feed intake and growth performance. Despite the differences in the CT concentrations of diets and in feed intake, CT intake was similar between lambs fed CL2.5 and Ex2.5 diets (26.9 and 28.1 g/day, respectively).

Inclusion of 125 g/kg of *C. ladanifer* aerial part in diet had a minor effect on DM and nutrient intake comparatively with unsupplemented diet, while for *C. ladanifer* CT extract at the same level of CT (Ex1.25) greater effects on the DM and nutrient intake were observed. However, such effects did not reflect in a different ADG and slaughter live weight between supplemented with 1.25% of CT and unsupplemented lambs.

Independently of the diets, feed intake and ADG observed in present experiment were lower than those obtained in previous study in which *C. ladanifer* was incorporated in high-forage diet (Jerónimo *et al.*, 2010). The lower intake and consequent lower growth performance of lambs could be due to heat stress which the animal suffer during present study. The present work was carried out between June 13 and July 24, 2017, and according to reports of national weather institute (Instituto Português do Mar e da Atmosfera, IPMA), the months of June and July were extremely hot and dry, observing in the weather station closest to the trial location, maximum air temperature values of 43.9 and 41.2 ºC, achieved on June 19 and July 3, respectively. In both months occurred a heat wave, a prolonged period of abnormally hot weather, during the periods from 16 to 21 June, 2 to 4 and 12 to 17 July (IPMA), which corresponded to 15 from the 35 days of the trial, and four of these days (from 16 to 19 June) coincided to the animal adaptation week to the dietary treatments. Animal productivity is highly affected by high ambient temperatures. Animal exposure to high ambient temperature develop adaptive mechanisms to reduce body heat, which involve increase of respiration rate and water consumption and reduction of feed intake (Marai, El-Darawany, Fadiel, & Abdel-Hafez, 2007). Reduction of the voluntary feed intake of essential nutrients result in decrease of the anabolic activity, which is reflected in lower growth performance (Marai *et al.*, 2007).

### 6.4.2. Carcass and meat traits

*Cistus ladanifer* inclusion in diet, at highest amount (CL2.5 diet), decreased the hot and cold carcass weight and supplementation of diets with 2.5% of *C. ladanifer* extract (Ex2.5 diet) led to carcasses with intermediate weights between CL2.5 and L diets, reflecting the effect of diets on feed intake and ADG. However, higher percentage of high price joints were observed in lambs fed CL2.5 and both diets with *C. ladanifer* CT extract comparatively with unsupplemented lambs. Such results differ from the previous studies with *C. ladanifer* incorporation in lamb diets (Francisco *et al.*, 2017; Francisco *et al.*, 2015; Jerónimo *et al.*, 2010), in which *C. ladanifer* did not affected carcass traits. Tissue composition of dissected cut was not affected by diets, but lambs fed CL2.5 diet presented a lower muscle/bone ratio, which resulted from the trend to higher bone proportion in these lambs. Although, the proportions of

intermuscular and subcutaneous fat did not differ among treatments, lambs from CL2.5 had lower numerical values than other lambs, which may contribute to higher bone proportion in these animals. Contrarily, in previous trail, Jerónimo *et al*. (2010) reported a significant increase in subcutaneous fat proportion and reduction of muscle in chump and shoulder cuts of lambs fed 250 g/kg of *C. ladanifer*. Such as in previous trial, inclusion of 250 g/kg of *C. ladanifer* increase ether extract content in diet, which could have led to a greater deposition of fat. However, the difference in results can be justify by reduction of feed intake, and consequently reduction of ether extract intake observed in present work.

Meat colour coordinates were not affected by inclusion of *C. ladanifer* in lamb diets in accordance with Jerónimo *et al*. (2010) and Francisco *et al*. (2015). Moreover, the *C. ladanifer* CT extract had minor effect on meat colour. In agreement with previous studies, *C. ladanifer* inclusion in diets did not affect meat cooking losses (Francisco *et al.*, 2017; Francisco *et al.*, 2015) and shear force (Francisco *et al.*, 2015). However, the use of *C. ladanifer* CT extract affected meat cooking losses and shear force, with higher cooking losses in Ex1.25 diet and higher shear force (more 9%) in both diets supplemented with *C. ladanifer* CT extract than in unsupplemented diet. Such results suggest that *C. ladanifer* CT extract may affect postmortem proteolysis of meat, affecting negatively its capacity to hold the water and tenderness (Lund, Heinonen, Baron, & Estevez, 2011). However, trained sensory panel was unable to detect such differences, and the sensory properties of meat did not differ among diets. In previous study, was reported that inclusion of *C. ladanifer* (up to 200 g/kg) increase slightly meat off-flavour intensity, however this was not observed in the present work.

### 6.4.3. Fatty acid composition of meat and subcutaneous fat

The main objective of the present work was to verify if incorporation of CT extract from *C. ladanifer* in oil supplemented diet is able to increase the contents of *t*11-18:1 and *c*9,*t*11-18:2 in lamb fat, such as previously observed when *C. ladanifer* aerial part was added to high-forage diet supplemented with blend of vegetable oils (Jerónimo *et al.*, 2010). Contrarily to previous results, inclusion of 250 g/kg of *C. ladanifer* aerial part in oil supplemented diet did not increase *t*11-18:1 and *c*9,*t*11-18:2 deposition in intramuscular fat or in subcutaneous fat. Among the diets with *C. ladanifer* CT (from plant aerial parts or from extract), only the supplementation with extract to achieve 1.25% of CT in diet (Ex1.25 diet) resulted in higher *t*11-18:1 content in intramuscular fat and subcutaneous fat than in diet without *C. ladanifer* CT supplementation. The Ex1.25 diet allowed increase *t*11-18:1 content in 25% in intramuscular fat and in 33% in subcutaneous fat, comparatively to L diet. Vaccenic acid (*t*11-18:1) is produced during BH of

18:2*n*-6 and 18:3*n*-3 in rumen (Harfoot & Hazlewood, 1997), whereby present results suggest that *C. ladanifer* CT extract, at level of 1.25% of CT in diet, increase ruminal production of *t*11-18:1. Condensed tannins have been reported as able to increase the ruminal production of *t*11-18:1, by inhibition of the last reductive step of BH, i.e. conversion of *t*11-18:1 to 18:0 (Khiaosa-Ard *et al.*, 2009; Vasta *et al.*, 2009b) or by increasing the initial steps of the BH (Carreño *et al.*, 2015; Guerreiro *et al.*, 2016a). Recent *in vitro* study, performed by our team, showed that the CT extract from *C. ladanifer* modulate ruminal BH, with accumulation of *t*11-18:1 and *c*9,*t*11-18:2 without inhibition of 18:0 production (Guerreiro *et al.*, 2016a). On this *in vitro* study was tested a higher CT level (6% of CT) than the used in Ex1.25 and Ex2.5 diet (1.25% and 2.5% of CT, respectively). However, in present study the highest CT dose did not affect *t*11-18:1 and *c*9,*t*11-18:2 contents in intramuscular and subcutaneous fat.

Increase of *t*11-18:1 ruminal production is desirable, because the main via of *c*9,*t*11-18:2 synthesis is through the endogenous conversion of *t*11-18:1 by Stearoyl-CoA desaturase (SCD) in tissues and mammary gland (Griinari & Bauman, 1999). However, despite the higher availability of *t*11-18:1 to endogenous synthesis of *c*9,*t*11-18:2, in intramuscular and subcutaneous fat from Ex1.25 diet *c*9,*t*11-18:2 contents was similar to L diet. Such result, may be due to downregulation of SCD activity and/or low fat deposition. The SCD is a key regulatory enzyme of adipogenesis (Sampath & Ntambi, 2011), and its activity is intrinsically related with fat deposition level (Bessa *et al.*, 2015). In fact, intramuscular fat content observed in present work was low (14.9 g/kg meat) and a fairly low lipid content was also found in subcutaneous fat (ranging from 362 to 624 mg/g of subcutaneous fat). In previous studies, with incorporation of several levels of *C. ladanifer* in oil supplemented lamb diets, intramuscular fat values of about 33-35 g/kg of meat were reported (Francisco *et al.*, 2017; Francisco *et al.*, 2015; Jerónimo *et al.*, 2010). Adipose tissue is composed mainly by triacylglycerides, and muscle lipids beyond triacylglycerides deposited mostly in adipocytes also comprise a significant proportion of phospholipids present in cell membranes (Scollan *et al.*, 2006; Wood *et al.*, 2008). While the structural phospholipids contents in muscle is relatively constant and independent of total fat content, the amount of triacylglycerides depends of the fat levels, being the increase of intramuscular fat content due to increase of the triacylglycerides (Scollan *et al.*, 2006; Wood *et al.*, 2008). Once, *c*9,*t*11-18:2 is deposited preferentially in triacylglycerides, in the lean animals the CLA content in meat is fairly low (Bessa *et al.*, 2015). Such as many others *trans*-FA produced during ruminal BH, *t*11-18:1 also is mainly deposited in triacylglycerides (Jerónimo *et al.*, 2011). In addition to increase of the *t*11-18:1 production in rumen, for maximizing *t*11-18:1 and *c*9,*t*11-18:2 in ruminant meat, it is essential to ensure greater fat deposition (Bessa *et al.*, 2015). The lower fat deposition observed in present trial might also be related with the high

environmental temperatures to which the animals were subjected, which can have led to decrease of the anabolic activity and increase in tissue catabolism (Marai *et al.*, 2007).

Although without statistical difference for all diets, the 18:0 content in both intramuscular and subcutaneous fat was higher in CT-enriched diets than in L diet, particularly in the diets with the highest levels of CT (CL2.5 and Ex2.5). Moreover, in subcutaneous fat from lambs fed CL2.5 and Ex2.5 diets the 18:0 was the second major FA, while in other diets 16:0 was the second most abundant FA. Conversely, in both intramuscular and subcutaneous fat was observed lower *c*9-18:1 content in CT-enriched diets than in L diet. Endogenous synthesis of monounsaturated FA, as *c*9-18:1 is catalyzed by the SCD through desaturation of 18:0 (Bessa *et al.*, 2015). So, higher 18:0 and lower *c*9-18:1 contents in the CT-enriched diets suggest a lower activity of the SCD. Contrary, in muscle from lambs fed diets supplemented with quebracho and *Terminalia chebula* extracts it was observed the increase of the SCD protein expression and activity, respectively (Rana, Tyagi, Hossain, & Tyagi, 2012; Vasta *et al.*, 2009c). The effect of the *C. ladanifer* on muscle SCD gene expression is still unclear (Francisco *et al.*, 2017; Francisco *et al.*, 2016).

## 6. 5. Conclusion

Our results indicate that inclusion of 1.25% of CT from *C. ladanifer* in oil supplemented forage diet allowed increase the deposition of *t*11-18:1 in intramuscular and subcutaneous fat. However, higher availability of *t*11-18:1 did not resulted in higher *c*9,*t*11-18:2 content in both tissues, which is probably related with a lower fat deposition. For this levels of *C. ladanifer* CT extract in diets, no relevant effects on growth performance, carcass traits and meat quality were found. Conversely, high amounts of aerial part or CT extract from *C. ladanifer* in diets had detrimental affects the growth performance, without beneficial effect on FA composition of intramuscular and subcutaneous fat. Such results showed that low levels of *C. ladanifer* CT extract may be used as a natural additive in ruminant diet as tool to induce beneficial changes in the ruminal BH pattern and consequently in the FA profile of its fat.

## Acknowledgements

The authors would like to thank Alentejo2020 program for financial support to the project "CistusRumen - Sustainable use of Rockrose (*Cistus ladanifer* L.) in small ruminants - Increase of the competitiveness and reduction of the environmental impact" (ALT20-03-0145-FEDER-000023) through the Regional Development European Fund. We also thank the Fundação para a Ciência e Tecnologia (FCT) through research grants to OG (SFRH/BD/84406/2012) and SPA (SFRH/BPD/76836/2011). The authors also like to thank Ana Paulino, Ana Rita Prazeres, Daniela Rosa, Flávia Fernandes, Ivone Torrado and Silvana Luz for cooperation in *Cistus ladanifer* harvest.

# CHAPTER 7

## GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The manipulation of FA composition on ruminant edible fats, in order to improve its nutritional value, through reduction of its SFA content, and increase of PUFA, namely CLA, is currently one of the main targets on ruminant production research. Dietary manipulation has shown to be effective means to improve FA composition of ruminant meat. The studies reported in this thesis aimed to further the knowledge regarding *C. ladanifer* nutritional composition, and its utilization on nutritional strategies to improve the nutritional value of lamb meat.

Previous work from our research group demonstrated that *C. ladanifer* inclusion in oil supplemented forage-based diets can be a promising strategy to enhance nutritional value of intramuscular fat, increasing its *c*9,*t*11-18:2 content (Jerónimo *et al.*, 2010). However, the information about *C. ladanifer* chemical and nutritional composition was very scarce. Therefore, the first aim was to acquire more knowledge about *C. ladanifer* chemical and nutritional composition, and as well as to assess its antioxidant activity. The seasonal variation of the *C. ladanifer* aerial part, from plants with two ages, was characterized for chemical composition, including the proximate composition and total phenols and CT contents, for *in vitro* digestibility, antioxidant activity and FA profile (Chapter 2 and 3). These two studies allowed to know the seasonal variation of chemical composition and nutritional value of *C. ladanifer* from both ages, information that to date was still very limited. Furthermore, it was possible to clarify, and to provide, a correct identification of *C. ladanifer* FA profile, being the aerial part composed mainly by SFA, identifying two BCFA, iso-19:0 and iso-21:0, detected for the first time in shrubs. Although widely available throughout the year, *C. ladanifer* is poorly grazed due to its low nutritional value and palatability. Indeed, *C. ladanifer* is considered as a nutritionally unbalanced feed, with moderate parietal compounds content, low protein levels, high CT contents and low organic matter digestibility, as it was confirmed in Chapter 2. However, we consider that *C. ladanifer* aerial part can be used in ruminant nutrition, but only associated with other feeding resources to complement its nutritional imbalances.

Recently, tannins have been explored as potential modulators of ruminal BH, and results from *in vitro* and *in vivo* studies indicate that tannins, either CT or hydrolysable tannins are able to influence the ruminal BH pattern (Carreño *et al.*, 2015; Costa *et al.*, 2017a; Khiaosa-Ard *et al.*, 2009; Vasta *et al.*, 2010b). Taking into account the high CT amount present in *C. ladanifer*, the previous results from our group were attributed to the effect of CT on ruminal BH. However, in addition to CT, *C. ladanifer* contains also high levels of other secondary compounds that might modulate ruminal BH. It was not clear what type of secondary compounds present in *C. ladanifer* might be responsible for its effects on rumen BH. So, effects of *C. ladanifer* fractions, rich in secondary metabolites, were tested on ruminal BH. Although several *C. ladanifer*

fractions had induced alterations upon FA profile, the CT fraction was the most active on ruminal BH modulation, leading to a higher accumulation of *t*11-18:1 and higher disappearance of the substrate PUFA, which suggested that the fractions promoted the BH initial steps (Chapter 4). These results support that CT from *C. ladanifer* might be used as natural additive on ruminant diets, as way to induce benefic alteration on the ruminal BH pattern and consequently on the FA profile of ruminant edible products. Further studies are needed for *C. ladanifer*, and its fractions, entire chemical characterization, in order to identify the specific compounds, or groups of compounds.

After identifying CT as modulator of ruminal BH, the level of CT inclusion in diets, which allow to optimize the *t*11-18:1 production, was not yet known. Therefore, we intended to determine which amount of CT can optimize the ruminal synthesis of *t*11-18:1. However, when increasing levels of CT fraction (up to 10% of extract, the same amount used on the first *in vitro* study), all CT levels led a decrease of the ruminal fermentation without effects on ruminal BH. Although this decrease was less pronounced with lower levels of CT (2.5 and 5% of CT extract) (Chapter 5). The tested extract was the same that previously used on the first study, nevertheless, several conditions of the incubation were changed (buffer solution, substrate and duration of incubation). So, it would be important to further understand what happened during the incubation, optimizing either the incubation conditions as well as CT levels which could increase the *t*11-18:1 production, without detrimental effects on ruminal fermentation.

The *in vitro* systems do not reflect the complexity of *in vivo* systems, being for this necessary the *in vivo* validation. Considering the results from the two *in vitro* studies, and our previous results, both *C. ladanifer* aerial part and its CT extract was incorporated on oil supplemented lamb diets. High levels of CT in diets are associated to detrimental effects in ruminants, namely in productive performance. So, we considered two levels of CT (1.25 and 2.5%), since 2.5% was the CT level of *C. ladanifer* inclusion on lamb diets, without compromise the productive performance (Jerónimo *et al.*, 2010). On the second *in vitro* study (chapter 5), the lowest levels of CT extract (corresponding to 1.5 and 3% of CT) induced lower alterations on ruminal fermentation. For that reason, we selected the CT levels of 1.25 and 2.5% for incorporation on ruminant diets, with two ways of CT supply (*C. ladanifer* aerial part and CT extract obtained from *C. ladanifer*) (Chapter 6). However, contrarily to the previous *in vivo* study the higher level of CT in diet (2.5%) affected the lamb productive performance, with decrease of average daily gain. Although, CT extract incorporation was less detrimental that *C. ladanifer* aerial part, for the highest CT level (2.5%). As the *C. ladanifer* have low nutritional value, the addition of 25% of *C. ladanifer* aerial part may have decreased diet nutritional value, and palatability, with

consequent intake decrease. The diet of 1.25% of CT extract (Ex1.25) led to the highest accumulation of *t*11-18:1 in tissues, and a similar amount of *c*9,*t*11-18:2 than control diet without CT inclusion. So, according to our objective, it seems that the Ex1.25 diet can be a good approach to increase the beneficial FA, i.e. *t*11-18:1 and *c*9,*t*11-18:2, on lamb meat.

The information about the interaction between CT and bacteria responsible for the BH process is scarce and controversial. So, further studies are needed to understand the effects of *C. ladanifer* plant, and its CT extracts, on the microbial ecosystem. This knowledge will contribute to elucidate the mechanisms by which *C. ladanifer*, and its CT extracts, are able to modulate the ruminal BH. Moreover, the increase of *t*11-18:1 ruminal production is desirable, because the main via of *c*9,*t*11-18:2 synthesis is through the endogenous conversion of *t*11-18:1 by SCD in tissues and mammary gland. So, it would be important to evaluate the effect of *C. ladanifer* aerial part and CT *C. ladanifer* extract on SCD gene and protein expression levels, and SCD activity in the muscle. Although *C. ladanifer* could be used as a natural additive in ruminant diet, its utilization requires great care, since the occurrence of metabolic disorders cannot be disregarded. Thus, the biochemical evaluation, when including *C. ladanifer* aerial part and CT *C. ladanifer* extract on ruminant diets, should be addressed.

At current knowledge, this was the first time in which a more detailed chemical and nutritional composition of a plant was evaluated with further fractionation into several extracts, rich in secondary compounds, in respect to ruminal BH. In the present work was possible to identify, *in vitro*, that *C. ladanifer* CT fraction is the most efficient on ruminal BH modulation. Although, in the second *in vitro* study it was not possible to determine which were the CT levels that optimize the accumulation of *t*11-18:1. On the productive trial, we obtained good results with 1.25 % of CT diet, since this was the only diet that induced FA profile enhancement, with accumulation of beneficial BI (*t*11-18:1 and *c*9,*t*11-18:2) on ruminant edible fats.

We can conclude that *C. ladanifer* CT extract may be a good approach to improve the nutritional value of the ruminant edible fats.

# REFERENCES

Acamovic, T., & Brooker, J. D. (2005). Biochemistry of plant secondary metabolites and their effects in animals. *Proceedings of the Nutrition Society, 64*(3), 403-412.

Agarwal, N., Shekhar, C., Kumar, R., Chaudhary, L. C., & Kamra, D. N. (2009). Effect of peppermint (*Mentha piperita*) oil on *in vitro* methanogenesis and fermentation of feed with buffalo rumen liquor. *Animal Feed Science and Technology, 148*(2-4), 321-327.

Aldai, N., de Renobales, M., Barron, L. J. R., & Kramer, J. K. G. (2013). What are the trans fatty acids issues in foods after discontinuation of industrially produced *trans* fats? Ruminant products, vegetable oils, and synthetic supplements. *European Journal of Lipid Science and Technology, 115*(12), 1378-1401.

Alexander, R., & Mcgowan, M. (1966). A filtration procedure for the *in vitro* determination of digestibility of herbage. *Journal of the British Grassland Society, 16*, 140-147.

Alfaia, C. P. M., Alves, S. P., Martins, S. I. V., Costa, A. S. H., Fontes, C. M. G. A., Lemos, J. P. C., et al. (2009). Effect of the feeding system on intramuscular fatty acids and conjugated linoleic acid isomers of beef cattle, with emphasis on their nutritional value and discriminatory ability. *Food Chemistry, 114*(3), 939-946.

Alías, J. C., Sosa, T., Valares, C., Escudero, J. C., & Chaves, N. (2012). Seasonal variation of *Cistus ladanifer* L. Diterpenes. *Plants, 1*(1), 6-15.

Alves, S. P., & Bessa, R. J. B. (2014). The *trans*-10,*cis*-15 18:2: a missing intermediate of *trans*-10 shifted rumen biohydrogenation pathway? [Article]. *Lipids, 49*(6), 527-541.

Alves, S. P., Bessa, R. J. B., Quaresma, M. A. G., Kilminster, T., Scanlon, T., Oldham, C., et al. (2013a). Does the fat tailed Damara ovine breed have a distinct lipid metabolism leading to a high concentration of branched chain fatty acids in tissues? [Article]. *Plos One, 8*(10).

Alves, S. P., Francisco, A., Costa, M., Santos-Silva, J., & Bessa, R. J. B. (2017). Biohydrogenation patterns in digestive contents and plasma of lambs fed increasing levels of a tanniferous bush (*Cistus ladanifer* L.) and vegetable oils. *Animal Feed Science and Technology, 225*, 157-172.

Alves, S. P., Santos-Silva, J., Cabrita, A. R. J., Fonseca, A. J. M., & Bessa, R. J. B. (2013b). Detailed dimethylacetal and fatty acid composition of rumen content from lambs fed lucerne or concentrate supplemented with soybean oil. *Plos One, 8*(3).

Ammar, H., Lopez, S., & Gonzalez, J. S. (2005). Assessment of the digestibility of some Mediterranean shrubs by *in vitro* techniques. *Animal Feed Science and Technology, 119*(3-4), 323-331.

Ammar, H., Lopez, S., Gonzalez, J. S., & Ranilla, M. J. (2004). Chemical composition and *in vitro* digestibility of some Spanish browse plant species. *Journal of the Science of Food and Agriculture, 84*(2), 197-204.

Andrade, D., Gil, C., Breitenfeld, L., Domingues, F., & Duarte, A. P. (2009). Bioactive extracts from *Cistus ladanifer* and *Arbutus unedo L. Industrial Crops and Products, 30*(1), 165-167.

Arrendale, R. F., Severson, R. F., Chortyk, O. T., & Stephenson, M. G. (1988). Isolation and Identification of the Wax Esters from the Cuticular Waxes of Green Tobacco Leaf. *Beitrage Zur Tabakforschung International, 14*(2), 67-84.

Association of Official Analytical Chemists. (1990). *Official Methods of Analysis* (15th AOAC ed.). Arlington, VA.

Barrajon-Catalan, E., Fernandez-Arroyo, S., Roldan, C., Guillen, E., Saura, D., Segura-Carretero, A., et al. (2011). A systematic study of the polyphenolic composition of aqueous extracts deriving from several *Cistus* genus species: evolutionary relationship. *Phytochem Anal*.

Barrajon-Catalan, E., Fernandez-Arroyo, S., Saura, D., Guillen, E., Fernandez-Gutierrez, A., Segura-Carretero, A., et al. (2010). Cistaceae aqueous extracts containing ellagitannins show antioxidant and antimicrobial capacity, and cytotoxic activity against human cancer cells. *Food and Chemical Toxicology, 48*(8-9), 2273-2282.

Barros, L., Duenas, M., Alves, C. T., Silva, S., Henriques, M., Santos-Buelga, C., et al. (2013). Antifungal activity and detailed chemical characterization of *Cistus ladanifer* phenolic extracts. *Industrial Crops and Products, 41*, 41-45.

Bastida, F., & Talavera, S. (2002). Temporal and spatial patterns of seed dispersal in two *Cistus* species (Cistaceae). [Article]. *Annals of Botany, 89*(4), 427-434.

Bauchart, D., Roy, A., Lorenz, S., Chardigny, J. M., Ferlay, A., Gruffat, D., et al. (2007). Butters varying in *trans* 18:1 and *cis*-9,*trans*-11 conjugated linoleic acid modify plasma lipoproteins in the hypercholesterolemic rabbit. [Article]. *Lipids, 42*(2), 123-133.

Bauman, D. E., Baumgard, L. H., Corl, B. A., & Griinari, d. J. M. (1999). *Biosynthesis of conjugated linoleic acid in ruminants.* Paper presented at the Proceedings of the American Society of Animal

Science.

Beam, T. M., Jenkins, T. C., Moate, P. J., Kohn, R. A., & Palmquist, D. L. (2000). Effects of amount and source of fat on the rates of lipolysis and biohydrogenation of fatty acids in ruminal contents. *Journal of Dairy Science, 83*(11), 2564-2573.

Belenguer, A., Hervas, G., Yanez-Ruiz, D. R., Toral, P. G., Ezquerro, C., & Frutos, P. (2010). Preliminary study of the changes in rumen bacterial populations from cattle intoxicated with young oak (*Quercus pyrenaica*) leaves. *Animal Production Science, 50*(3), 228-234.

Belmokhtar, M., Bouanani, N. E., Ziyyat, A., Mekhfi, H., Bnouham, M., Aziz, M., et al. (2009). Antihypertensive and endothelium-dependent vasodilator effects of aqueous extract of *Cistus ladaniferus*. [Article]. *Biochemical and Biophysical Research Communications, 389*(1), 145-149.

Benchaar, C., McAllister, T. A., & Chouinard, P. Y. (2008). Digestion, ruminal fermentation, ciliate protozoa populations, and milk production from dairy cows fed cinnamaldehyde, quebracho condensed tannins, or *Yucca schigadera* saponin extracts. *Journal of Dairy Science, 91*, 4765-4777.

Bessa, R. J. B., Alves, S. P., Jeronimo, E., Alfaia, C. M., Prates, J. A. M., & Santos-Silva, J. (2007). Effect of lipid supplements on ruminal biohydrogenation intermediates and muscle fatty acids in lambs. *European Journal of Lipid Science and Technology, 109*(8), 868-878.

Bessa, R. J. B., Alves, S. P., & Santos-Silva, J. (2015). Constraints and potentials for the nutritional modulation of the fatty acid composition of ruminant meat. [Review]. *European Journal of Lipid Science and Technology, 117*(9), 1325-1344.

Bessa, R. J. B., Maia, M. R. G., Jeronimo, E., Belo, A. T., Cabrita, A. R. J., Dewhurst, R. J., et al. (2009). Using microbial fatty acids to improve understanding of the contribution of solid associated bacteria to microbial mass in the rumen. [Article]. *Animal Feed Science and Technology, 150*(3-4), 197-206.

Bessa, R. J. B., Portugal, P. V., Mendes, I. A., & Santos-Silva, J. (2005). Effect of lipid supplementation on growth performance, carcass and meat quality and fatty acid

composition of intramuscular lipids of lambs fed dehydrated lucerne or concentrate. *Livestock Production Science, 96*(2-3), 185-194.

Bessa, R. J. B., Santos-Silva, J., Ribeiro, J. M. R., & Portugal, A. V. (2000). Reticulo-rumen biohydrogenation and the enrichment of ruminant edible products with linoleic acid conjugated isomers. *Livestock Production Science, 63*(3), 201-211.

Bhat, T. K., Singh, B., & Sharma, O. P. (1998). Microbial degradation of tannins - A current perspective. *Biodegradation, 9*(5), 343-357.

Blondel, J. (2006). The 'design' of mediterranean landscapes: A millennial story of humans and ecological systems during the historic period. *Human Ecology, 34*(5), 713-729.

Bruno-Soares, A. M., Abreu, J. M. F., Guedes, C. V. M., & Dias-da-Silva, A. A. (2000). Chemical composition, DM and NDF degradation kinetics in rumen of seven legume straws. *Animal Feed Science and Technology, 83*(1), 75-80.

Bruno-Soares, A. M., Cadima, J., & Matos, T. D. S. (2010). Predicting degradability parameters of diets for ruminants using regressions on chemical components. [Article]. *Journal of the Science of Food and Agriculture, 90*(6), 949-955.

Bruno-Soares, A. M., Ferreira, J. P., Sousa, E., & Abreu, J. M. (1999). As Cistáceas nas Pastagens Naturais do Alentejo. I – Efeitos da Ingestão de *Cistus salvifolius* por Ovinos (The Cistaceae in Natural Pastures of Alentejo. I - Effects of Ingestion of *Cistus salvifolius* by ovines). *Pastagens e Forragens, 20*, 21-32.

Bruno-Soares, A. M., Matos, T. J. S., & Cadima, J. (2011). Nutritive value of *Cistus salvifolius* shrubs for small ruminants. *Animal Feed Science and Technology, 165*(3-4), 167-175.

Bu, D. P., Wang, J. Q., Dhiman, T. R., & Liu, S. J. (2007). Effectiveness of oils rich in linoleic and linolenic acids to enhance conjugated linoleic acid in milk from dairy cows. *Journal of Dairy Science, 90*(2), 998-1007.

Buccioni, A., Decandia, M., Minieri, S., Molle, G., & Cabiddu, A. (2012). Lipid metabolism in the rumen: New insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors. *Animal Feed Science and Technology, 174*(1), 1-25.

Buccioni, A., Minieri, S., Rapaccini, S., Antongiovanni, M., & Mele, M. (2011). Effect of chestnut and quebracho tannins on fatty acid profile in rumen liquid- and solid-associated bacteria: an *in vitro* study. *Animal, 5*(10), 1521-1530.

Buccioni, A., Pallara, G., Pastorelli, R., Bellini, L., Cappucci, A., Mannelli, F., et al. (2017). Effect of dietary chestnut or quebracho tannin supplementation on microbial community and fatty acid profile in the rumen of dairy ewes. *BioMed Research International*.

Buccioni, A., Serra, A., Minieri, S., Mannelli, F., Cappucci, A., Benvenuti, D., et al. (2015). Milk production, composition, and milk fatty acid profile from grazing sheep fed diets supplemented with chestnut tannin extract and extruded linseed. *Small Ruminant Research, 130*, 200-207.

Cabezudo, B., Navarro, T., Pérez Latorre, A., Nieto Caldera, J. M., & Orshan, G. (1992). Estudios fenomorfológicos en la vegetación del sur de España. I. *Cistus L.* (Phenomorphologic studies in vegetation of South of Spain. I. Cistus *L.*). *Acta Botanica Malacitana, 17*, 229-237.

Cabiddu, A., Molle, G., Decandia, M., Spada, S., Fiori, M., Piredda, G., et al. (2009). Responses to condensed tannins of flowering sulla (*Hedysarum coronarium* L.) grazed by dairy sheep Part 2: Effects on milk fatty acid profile. *Livestock Science, 123*(2-3), 230-240.

Calder, P. C. (2015). Functional roles of fatty acids and their effects on human health. *Journal of Parenteral and Enteral Nutrition, 39*(1_suppl), 18S-32S.

Calder, P. C. (2017). Very long-chain n-3 fatty acids and human health: fact, fiction and the future. *Proceedings of the Nutrition Society*, 1-21.

Calsamiglia, S., Busquet, M., Cardozo, P. W., Castillejos, L., & Ferret, A. (2007). Essential oils as modifiers of rumen microbial fermentation. *Journal of Dairy Science, 90*(6), 2580-2595.

Carreño, D., Hervas, G., Toral, P. G., Belenguer, A., & Frutos, P. (2015). Ability of different types and doses of tannin extracts to modulate in vitro ruminal biohydrogenation in sheep. *Animal Feed Science and Technology, 202*, 42-51.

Castagnino, P. S., Messana, J. D., Fiorentini, G., de Jesus, R. B., Vito, E. S., Carvalho, I. P. C., et al. (2015). Glycerol combined with oils did not limit biohydrogenation of unsaturated fatty acid but reduced methane production *in vitro*. [Article]. *Animal Feed Science and Technology, 201*, 14-24.

Castro-Carrera, T., Toral, P. G., Frutos, P., McEwan, N. R., Hervas, G., Abecia, L., et al. (2014). Rumen bacterial community evaluated by 454 pyrosequencing and terminal restriction fragment length polymorphism analyses in dairy sheep fed marine algae. *Journal of Dairy Science, 97*(3), 1661-1669.

Chardigny, J. M., Destaillats, F., Malpuech-Brugere, C., Moulin, J., Bauman, D. E., Lock, A. L., et al. (2008). Do *trans* fatty acids from industrially produced sources and from natural sources have the same effect on cardiovascular disease risk factors in healthy subjects? Results of the *trans* fatty acids collaboration (TRANSFACT) study. [Article]. *American Journal of Clinical Nutrition, 87*(3), 558-566.

Chaves, N., Escudero, J. C., & Gutiérrez-merino, C. (1993). Seasonal-Variation of Exudate of Cistus-Ladanifer. *Journal of Chemical Ecology, 19*(11), 2577-2591.

Chaves, N., Escudero, J. C., & Gutiérrez-Merino, C. (1997a). Role of ecological variables in the seasonal variation of flavonoid content of *Cistus ladanifer* exudate. *Journal of Chemical Ecology, 23*(3), 579-603.

Chaves, N., Escudero, J. C., & Gutierrez Merino, C. (1997b). Quantitative variation of flavonoids among individuals of a *Cistus ladanifer* population. *Biochemical Systematics and Ecology, 25*(5), 429-435.

Chaves, N., Sosa, T., Alias, J. C., & Escudero, J. C. (2001). Identification and effects of interaction phytotoxic compounds from exudate of *Cistus ladanifer* leaves. *Journal of Chemical Ecology, 27*(3), 611-621.

Chikwanha, O. C., Vahmani, P., Muchenje, V., Dugan, M. E. R., & Mapiye, C. (2017). Nutritional enhancement of sheep meat fatty acid profile for human health and wellbeing. *Food Research International*.

Chilliard, Y., Glasser, F., Ferlay, A., Bernard, L., Rouel, J., & Doreau, M. (2007). Diet, rumen biohydrogenation and nutritional quality of cow and goat milk fat. *European Journal of Lipid Science and Technology, 109*(8), 828-855.

Cook, L. J., Scott, T. W., Ferguson, K. A., & McDonald, I. W. (1970). Production of poly-unsaturated ruminant body fats. [Article]. *Nature, 228*(5267), 178-&.

Cosentino, S. L., Gresta, F., & Testa, G. (2014). Forage chain arrangement for sustainable livestock systems in a Mediterranean area. *Grass and Forage Science, 69*(4), 625-634.

Costa, M., Alves, S. P., Cabo, A., Guerreiro, O., Stilwell, G., Dentinho, M. T., et al. (2017a). Modulation of *in vitro* rumen biohydrogenation by *Cistus ladanifer* tannins compared with other tannin sources. *Journal of the Science of Food and Agriculture, 97*(2), 629-635.

Costa, M., Alves, S. P., Francisco, A., Almeida, J., Alfaia, C. M., Martins, S. V., et al. (2017b). The reduction of starch in finishing diets supplemented with oil does not prevent the

accumulation of *trans*-10 18:1 in lamb meat. [Article]. *Journal of Animal Science, 95*(8), 3745-3761.

Dai, J., & Mumper, R. J. (2010). Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. [Review]. *Molecules, 15*(10), 7313-7352.

David, J. P., Ferrari, J., David, J. M., Guimaraes, A. G., Lima, F. W. D. M., & de Souza, G. L. S. (2007). New triterpene and antibacterial labdenoic acid derivatives from *Moldenhawera nutans*. *Journal of the Brazilian Chemical Society, 18*(8), 1585-1589.

Dawson, R. M. C., Hemington, N., & Hazlewood, G. P. (1977). On the role of higher plant and microbial lipases in the ruminal hydrolysis of grass lipids. *British Journal of Nutrition, 38*(2), 225-232.

Dawson, R. M. C., & Kemp, P. (1969). Effect of defaunation on phospholipids and on hydrogenation of unsaturated fatty acids in rumen. *Biochemical Journal, 115*(2), 351-&.

De Smet, S., & Vossen, E. (2016). Meat: The balance between nutrition and health. A review. [Article; Proceedings Paper]. *Meat Science, 120*, 145-156.

Degen, A. A., Blanke, A., Becker, K., Kam, M., Benjamin, R. W., & Makkar, H. P. S. (1997). The nutritive value of *Acacia saligna* and *Acacia salicina* for goats and sheep. *Animal Science, 64*, 253-259.

Demeyer, D. I., Henderson, C., & Prins, R. A. (1978). Relative significance of exogenous and *de novo* synthesized fatty acids in formation of rumen microbial lipids *in vitro*. *Applied and Environmental Microbiology, 35*(1), 24-31.

Demoly, J. P., & Montserrat, P. (1993). *Cistus*. *Flora iberica, 3*, 319-337.

Dentinho, M. T. P., Belo, A. T., & Bessa, R. J. B. (2014). Digestion, ruminal fermentation and microbial nitrogen supply in sheep fed soybean meal treated with *Cistus ladanifer* L. tannins. *Small Ruminant Research, 119*(1-3), 57-64.

Dentinho, M. T. P., Moreira, O. C., Pereira, M. S., & Bessa, R. J. B. (2007). The use of a tannin crude extract from *Cistus ladanifer L*. to protect soya-bean protein from degradation in the rumen. *Animal, 1*(5), 645-650.

Dentinho, M. T. P., Navas, D., & Potes, J. (2005). Chemical and nutritional evaluation of food complements for large cattle breeding, in Montado de azinho area. *Pastagens e Forragens, 26/27*, 41-46.

Depascualt, J., Bellido, I. S., Basabe, P., Marcos, I. S., Ruano, L. F., & Urones, J. G. (1982). Labdane Diterpenoids from *Cistus-Ladaniferus*. *Phytochemistry, 21*(4), 899-901.

Destaillats, F., Trottier, J. P., Galvez, J. M. G., & Angers, P. (2005). Analysis of alpha-linolenic acid biohydrogenation intermediates in milk fat with emphasis on conjugated linolenic acids. *Journal of Dairy Science, 88*(9), 3231-3239.

Devillard, E., McIntosh, F. M., Newbold, C. J., & Wallace, R. J. (2006). Rumen ciliate protozoa contain high concentrations of conjugated linoleic acids and vaccenic acid, yet do not hydrogenate linoleic acid or desaturate stearic acid. *British Journal of Nutrition, 96*(4), 697-704.

Dias, L. S., & Moreira, I. (2002). Interaction between water soluble and volatile compounds of *Cistus ladanifer* L. *Chemoecology, 12*(2), 77-82.

Diaz, A., Ranilla, M. J., Giraldo, L. A., Tejido, M. L., & Carro, M. D. (2015). Treatment of tropical forages with exogenous fibrolytic enzymes: effects on chemical composition and *in vitro* rumen fermentation. [Article]. *Journal of Animal Physiology and Animal Nutrition, 99*(2), 345-355.

Dilzer, A., & Park, Y. (2012). Implication of conjugated linoleic acid (CLA) in human health. *Critical Reviews in Food Science and Nutrition, 52*(6), 488-513.

Domenech-Carbo, M. T., de la Cruz-Canizares, J., Osete-Cortina, L., Domenech-Carbo, A., & David, H. (2009). Ageing behaviour and analytical characterization of the Jatoba resin collected from *Hymenaea stigonocarpa* Mart. *International Journal of Mass Spectrometry, 284*(1-3), 81-92.

Doreau, M., & Chilliard, Y. (1997). Digestion and metabolism of dietary fat in farm animals. *British Journal of Nutrition, 78*(1), S15-S35.

Doreau, M., & Ferlay, A. (1994). Digestion and utilization of fatty acids by ruminants. *Animal Feed Science and Technology, 45*(3-4), 379-396.

Drackley, J. K. (2000). Lipid metabolism. *Farm animal metabolism and nutrition*, 97-119.

Durmic, Z., McSweeney, C. S., Kemp, G. W., Hutton, P., Wallace, R. J., & Vercoe, P. E. (2008). Australian plants with potential to inhibit bacteria and processes involved in ruminal biohydrogenation of fatty acids. *Animal Feed Science and Technology, 145*(1-4), 271-284.

EFSA. (2010). Scientific opinion on dietary reference values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, *trans* fatty acids, and cholesterol. *European Fodd and Safety Authority Journal, 8*, 1-107.

El Youbi, A. E., El Mansouri, L., Boukhira, S., Daoudi, A., & Bousta, D. (2016). *In Vivo* Anti-Inflammatory and Analgesic Effects of Aqueous Extract of *Cistus ladanifer* L. From Morocco. [Article]. *American Journal of Therapeutics, 23*(6), E1554-E1559.

Enjalbert, F., Eynard, P., Nicot, M. C., Troegeler-Meynadier, A., Bayourthe, C., & Moncoulon, R. (2003). *In vitro* versus *in situ* ruminal biohydrogenation of unsaturated fatty acids from a raw or extruded mixture of ground canola seed/canola meal. [Article]. *Journal of Dairy Science, 86*(1), 351-359.

Enzell, C. R., & Ryhage, R. (1965). Mass Spectrometric Studies of Diterpenes 1 Carbodicyclic Diterpenes. *Arkiv for Kemi, 23*(5), 367-&.

Falcone, D., Ogas, J., & Somerville, C. (2004). Regulation of membrane fatty acid composition by temperature in mutants of *Arabidopsis* with alterations in membrane lipid composition. *BMC Plant Biology, 4*, 17.

Falleh, H., Ksouri, R., Chaieb, K., Karray-Bouraoui, N., Trabelsi, N., Boulaaba, M., et al. (2008). Phenolic composition of *Cynara cardunculus L*. organs, and their biological activities. *Comptes Rendus Biologies, 331*(5), 372-379.

FAO. (2010). *Fats and fatty acids in human nutrition: report of an expert consultation* (Vol. 91). Rome, Italy.

Ferlay, A., Bernard, L., Meynadier, A., & Malpuech-Brugere, C. (2017). Production of *trans* and conjugated fatty acids in dairy ruminants and their putative effects on human health: A review. [Review]. *Biochimie, 141*, 107-120.

Fernandez-Arroyo, S., Barrajon-Catalan, E., Micol, V., Segura-Carretero, A., & Fernandez-Gutierrez, A. (2010). High-performance liquid chromatography with diode array detection coupled to electrospray time-of-flight and ion-trap tandem mass spectrometry to identify phenolic compounds from a *Cistus ladanifer* aqueous extract. *Phytochemical Analysis, 21*(4), 307-313.

Ferrandis, P., Herranz, J. M., & Martinez-Sanchez, J. J. (1999). Effect of fire on hard-coated Cistaceae seed banks and its influence on techniques for quantifying seed banks. *Plant Ecology, 144*(1), 103-114.

Ferreira, S., Santos, J., Duarte, A., Duarte, A. P., Queiroz, J. A., & Domingues, F. C. (2011). Screening of antimicrobial activity of *Cistus ladanifer* and *Arbutus unedo* extracts. [Article]. *Natural Product Research, 26*(16), 1558-1560.

Foiklang, S., Wanapat, M., & Norrapoke, T. (2016). Effect of Grape Pomace Powder, Mangosteen Peel Powder and Monensin on Nutrient Digestibility, Rumen Fermentation, Nitrogen Balance and Microbial Protein Synthesis in Dairy Steers. *Asian-Australasian Journal of Animal Sciences, 29*(10), 1416-1423.

Fonseca, A. J. M., Cabrita, A. R. J., Lage, A. M., & Gomes, E. (2000). Evaluation of the chemical composition and the particle size of maize silages produced in north-west of Portugal. *Animal Feed Science and Technology, 83*(3-4), 173-183.

Francisco, A., Alves, S. P., Portugal, P. V., Dentinho, M. T., Jerónimo, E., Sengo, S., et al. (2017). Effects of dietary inclusion of citrus pulp and rockrose soft stems and leaves on lamb meat quality and fatty acid composition. *Animal*, 1-10 (In Press).

Francisco, A., Alves, S. P., Portugal, P. V., Pires, V. M. R., Dentinho, M. T., Alfaia, C. M., et al. (2016). Effect of feeding lambs with a tanniferous shrub (rockrose) and a vegetable oil blend on fatty acid composition of meat lipids. [Article]. *Animal, 10*(12), 2061-2073.

Francisco, A., Dentinho, M. T., Alves, S. P., Portugal, P. V., Fernandes, F., Sengo, S., et al. (2015). Growth performance, carcass and meat quality of lambs supplemented with increasing levels of a tanniferous bush (*Cistus ladanifer* L.) and vegetable oils. *Meat Sci, 100*, 275-282.

Frazão, D. F., Raimundo, J. R., Domingues, J. L., Quintela-Sabarís, C., Gonçalves, J. C., & Delgado, F. (2017). *Cistus ladanifer* (Cistaceae): a natural resource in Mediterranean-type ecosystems. *Planta*, 1-12.

Frutos, P., Hervas, G., Giraldez, F. J., & Mantecon, A. R. (2004). An *in vitro* study on the ability of polyethylene glycol to inhibit the effect of quebracho tannins and tannic acid on rumen fermentation in sheep, goats, cows, and deer. *Australian Journal of Agricultural Research, 55*(11), 1125-1132.

Frutos, P., Hervás, G., Giráldez, F. J., & Mantecón, A. R. (2004). Review. Tannins and ruminant nutrition. *Spanish Journal of Agricultural Research, 2*(2), 191-202.

Fulco, A. J. (1983). Fatty acid metabolism in bacteria. *Progress in Lipid Research, 22*(2), 133-160.

Garton, G. A., Vioque, E., & Lough, A. K. (1961). Glyceride hydrolysis and glycerol fermentation by sheep rumen contents. *Journal of General Microbiology, 25*(2), 215-&.

Gebauer, S. K., Destaillats, F., Mouloungui, Z., Candy, L., Bezelgues, J. B., Dionisi, F., et al. (2011). Effect of *trans* fatty acid isomers from ruminant sources on risk factors of cardiovascular disease: Study design and rationale. [Article]. *Contemporary Clinical Trials, 32*(4), 569-576.

Givens, D. I. (2009). Animal nutrition and lipids in animal products and their contribution to human intake and health. [Review]. *Nutrients, 1*(1), 71-82.

Givens, D. I. (2010). Milk and meat in our diet: good or bad for health? [Article]. *Animal, 4*(12), 1941-1952.

Glasser, F., Doreau, M., Maxin, G., & Baumont, R. (2013). Fat and fatty acid content and composition of forages: A meta-analysis. [Article]. *Animal Feed Science and Technology, 185*(1-2), 19-34.

Goering, H. K., & Van Soest, P. J. (1970). Forage fiber analyses (apparatus, reagents, procedures, and some applications). In ARS-USDA (Ed.), *Agricultural Research Service Handbook nº 379*. Washington, DC.

Gokkus, A., Parlak, A. O., & Parlak, M. (2011). Change of mineral element content in the common shrubs of Mediterranean zone. I. Macronutrients. *Zemdirbyste-Agriculture, 98*(4), 357-366.

Gomes, P. B., Mata, V. G., & Rodrigues, A. E. (2005). Characterization of the Portuguese-grown *Cistus ladanifer* essential oil. *Journal of Essential Oil Research, 17*(2), 160-165.

Gravador, R. S., Luciano, G., Jongberg, S., Bognanno, M., Scerra, M., Andersen, M. L., et al. (2015). Fatty acids and oxidative stability of meat from lambs fed carob-containing diets. *Food Chemistry, 182*, 27-34.

Greche, H., Mrabet, N., Zrira, S., Ismaili-Alaoui, M., Benjilali, B., & Boukir, A. (2009). The volatiles of the leaf oil of *Cistus ladanifer* L. var. *albiflorus* and labdanum extracts of Moroccan origin and their antimicrobial activities. [Article]. *Journal of Essential Oil Research, 21*(2), 166-173.

Griinari, J. M., & Bauman, D. E. (1999). Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants. *Advances in conjugated linoleic acid research, 1*, 180-200.

Griinari, J. M., Corl, B. A., Lacy, S. H., Chouinard, P. Y., Nurmela, K. V. V., & Bauman, D. E. (2000). Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Delta(9)-desaturase. *Journal of Nutrition, 130*(9), 2285-2291.

Guerreiro, O., Alves, S. P., Costa, M., Cabo, A., Duarte, M. F., Jerónimo, E., et al. (2016a). Effects of extracts obtained from *Cistus ladanifer* L. on *in vitro* rumen biohydrogenation. *Animal Feed Science and Technology, 219*, 304-312.

Guerreiro, O., Alves, S. P., Duarte, M. F., Bessa, R. J. B., & Jerónimo, E. (2015). *Cistus ladanifer* L. shrub is rich in saturated and branched chain fatty acids and their concentration increases in the Mediterranean dry season. *Lipids, 50*(5), 493-501.

Guerreiro, O., Dentinho, M. T., Moreira, O. C., Guerra, A. R., Ramos, P. A. B., Bessa, R. J. B., et al. (2016b). Potential of *Cistus ladanifer* L. (rockrose) in small ruminant diets - effect of season and plant age on chemical composition, *in vitro* digestibility and antioxidant activity. *Grass and Forage Science, 71*, 437-447.

Guil-Guerrero, J. L. (2014). Common mistakes about fatty acids identification by gas-liquid chromatography. *Journal of Food Composition and Analysis, 33*(2), 153-154.

Guimarães, R., Barros, L., Carvalho, A. M., Sousa, M. J., Morais, J. S., & Ferreira, I. C. F. R. (2009). Aromatic plants as a source of important phytochemicals: Vitamins, sugars and fatty acids in *Cistus ladanifer*, *Cupressus lusitanic*a and *Eucalyptus gunnii* leaves. *Industrial Crops and Products, 30*(3), 427-430.

Gunal, M., Ishlak, A., & Abughazaleh, A. A. (2013). Evaluating the effects of six essential oils on fermentation and biohydrogenation in *in vitro* rumen batch cultures. *Czech Journal of Animal Science, 58*(6), 243-252.

Gunal, M., Ishlak, A., AbuGhazaleh, A. A., & Khattab, W. (2014). Essential oils effect on rumen fermentation and biohydrogenation under *in vitro* conditions. *Czech Journal of Animal Science, 59*(10), 450-459.

Gutterman, Y. (1994). Strategies of seed dispersal and germination in plants inhabiting deserts. [Review]. *Botanical Review, 60*(4), 373-425.

Guzman, B., & Vargas, P. (2005). Systematics, character evolution, and biogeography of *Cistus* L. (Cistaceae) based on ITS, trnL-trnF, and matK sequences. [Article]. *Molecular Phylogenetics and Evolution, 37*(3), 644-660.

Guzman, B., & Vargas, P. (2009). Long-distance colonization of the Western Mediterranean by *Cistus ladanifer* (Cistaceae) despite the absence of special dispersal mechanisms. [Article]. *Journal of Biogeography, 36*(5), 954-968.

Hackmann, T. J., & Firkins, J. L. (2015). Maximizing efficiency of rumen microbial protein production. [Review]. *Frontiers in Microbiology, 6*, 16.

Hagerman, A. E. (1992). Tannin-protein interactions. In H. C. a. H. MT (Ed.), *Phenolic compounds in food and their effects on Health. I. Analysis, occurrence and chemistry* (pp. 236-247). Washington, DC: ACS Publications.

Hagerman, A. E. (2002). Tannin chemistry. In M. University (Ed.), *Tannin Handbook.* Miami: Oxford.

Hagerman, A. E., Robbins, C. T., Weerasuriya, Y., Wilson, T. C., & Mcarthur, C. (1992). Tannin Chemistry in Relation to Digestion. *Journal of Range Management, 45*(1), 57-62.

Harfoot, C. G., & Hazlewood, G. P. (1997). Lipid metabolism in the rumen *The rumen microbial ecosystem* (pp. 382-426): Springer.

Harfoot, C. G., Noble, R. C., & Moore, J. H. (1973). Factors influencing extent of biohydrogenaion of linoleic acid by rumen microorganisms *in vitro*. [Article]. *Journal of the Science of Food and Agriculture, 24*(8), 961-970.

Hatano, T., Kagawa, H., Yasuhara, T., & Okuda, T. (1988). Two new flavonoids and other constituents in licorice root - their relative astringency and radical scavenging effects. *Chemical & Pharmaceutical Bulletin, 36*(6), 2090-2097.

Hatew, B., Stringano, E., Mueller-Harvey, I., Hendriks, W. H., Carbonero, C. H., Smith, L. M. J., et al. (2016). Impact of variation in structure of condensed tannins from sainfoin (*Onobrychis viciifolia*) on *in vitro* ruminal methane production and fermentation characteristics. *Journal of Animal Physiology and Animal Nutrition, 100*(2), 348-360.

Honkanen, A. M., Griinari, J. M., Vanhatalo, A., Ahvenjarvi, S., Toivonen, V., & Shingfield, K. J. (2012). Characterization of the disappearance and formation of biohydrogenation intermediates during incubations of linoleic acid with rumen fluid in vitro. *Journal of Dairy Science, 95*(3), 1376-1394.

Hudson, J. A., MacKenzie, C. A. M., & Joblin, K. N. (1995). Conversion of oleic acid to 10-hydroxystearic acid by two species of ruminal bacteria. *Applied Microbiology and Biotechnology, 44*(1-2), 1-6.

Huws, S. A., Kim, E. J., Lee, M. R. F., Scott, M. B., Tweed, J. K. S., Pinloche, E., et al. (2011). As yet uncultured bacteria phylogenetically classified as *Prevotella*, *Lachnospiraceae incertae sedis* and unclassified *Bacteroidales*, *Clostridiales* and *Ruminococcaceae* may play a predominant role in ruminal biohydrogenation. *Environmental Microbiology*, *13*(6), 1500-1512.

ISO 6491. Animal feeding stuffs - determination of phosphorus content - spectrometric method (1995).

ISO 5983. Animal feeding stuffs–Determination of nitrogen content and calculation of crude protein content–Kjeldhal method. (1997).

ISO 9831. Animal feeding stuffs, animal products and faeces or urine - determination of gross calorimetric value - Bomb calorimeter method (1998).

ISO 6492. Animal feeding stuffs - Determination of fat content (1999a).

ISO 6496. Animal feeding stuffs - determination of moisture and the other volatile matter content (1999b).

ISO 6869. Animal feeding stuffs - determination of the contents of calcium, copper, iron, magnesium, manganese, potassium, sodium and zinc - method using absorption spectrometry (2000).

ISO 5984. Animal feeding stuffs – Determination of crude ash (2002).

IPMA. Instituto Português do Mar e da Atmosfera. Retrieved 30 January 2018: http://www.ipma.pt/pt/publicacoes/boletins.jsp?cmbDep=cli&cmbTema=pcl&cmbAno=2017&idDep=cli&idTema=pcl&curAno=2017

Jafari, S., Goh, Y. M., Rajion, M. A., Jahromi, M. F., Ahmad, Y. H., & Ebrahimi, M. (2017). Papaya (*Carica papaya*) leaf methanolic extract modulates *in vitro* rumen methanogenesis and rumen biohydrogenation. *Animal Science Journal*, *88*(2), 267-276.

Jafari, S., Goh, Y. M., Rajion, M. A., Jahromi, M. F., & Ebrahimi, M. (2016a). Ruminal methanogenesis and biohydrogenation reduction potential of papaya (*Carica papaya*) leaf: an *in vitro* study. *Italian Journal of Animal Science*, *15*(1), 157-165.

Jafari, S., Meng, G. Y., Rajion, M. A., Jahromi, M. F., & Ebrahimi, M. (2016b). Manipulation of Rumen Microbial Fermentation by Polyphenol Rich Solvent Fractions from Papaya Leaf to Reduce Green-House Gas Methane and Biohydrogenation of C18 PUFA. [Article]. *Journal of Agricultural and Food Chemistry*, *64*(22), 4522-4530.

Jayanegara, A., Kreuzer, M., Wina, E., & Leiber, F. (2011). Significance of phenolic compounds in tropical forages for the ruminal bypass of polyunsaturated fatty acids and the appearance of biohydrogenation intermediates as examined *in vitro*. *Animal Production Science, 51*(12), 1127-1136.

Jenkins, T. C. (2010). Technical note: Common analytical errors yielding inaccurate results during analysis of fatty acids in feed and digesta samples. *Journal of Dairy Science, 93*(3), 1170-1174.

Jenkins, T. C., AbuGhazaleh, A. A., Freeman, S., & Thies, E. J. (2006). The production of 10-hydroxystearic and 10-ketostearic acids is an alternative route of oleic acid transformation by the ruminal microbiota in cattle. *Journal of Nutrition, 136*(4), 926-931.

Jenkins, T. C., Wallace, R. J., Moate, P. J., & Mosley, E. E. (2008). BOARD-INVITED REVIEW: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. *Journal of Animal Science, 86*(2), 397-412.

Jerónimo, E., Alfaia, C. M. M., Alves, S. P., Dentinho, M. T. P., Prates, J. A. M., Vasta, V., et al. (2012). Effect of dietary grape seed extract and *Cistus ladanifer L*. in combination with vegetable oil supplementation on lamb meat quality. *Meat Science, 92*(4), 841-847.

Jerónimo, E., Alves, S. P., Alfaia, C. M., Prates, J. A. M., Santos-Silva, J., & Bessa, R. J. B. (2011). Biohydrogenation intermediates are differentially deposited between polar and neutral intramuscular lipids of lambs. *European Journal of Lipid Science and Technology, 113*(7), 924-934.

Jerónimo, E., Alves, S. P., Dentinho, M. T., Martins, S. V., Prates, J. A., Vasta, V., et al. (2010). Effect of grape seed extract, *Cistus ladanifer L.*, and vegetable oil supplementation on fatty acid composition of abomasal digesta and intramuscular fat of lambs. *Journal of Agricultural and Food Chemistry, 58*(19), 10710-10721.

Jerónimo, E., Alves, S. P., Prates, J. A. M., Santos-Silva, J., & Bessa, R. J. B. (2009). Effect of dietary replacement of sunflower oil with linseed oil on intramuscular fatty acids of lamb meat. [Article]. *Meat Science, 83*(3), 499-505.

Jones, G. A., McAllister, T. A., Muir, A. D., & Cheng, K. J. (1994). Effects of sainfoin (*Onobrychis viciifolia* Scop.) condensed tannins on growth and proteolysis by four strains of ruminal bateria. *Applied and Environmental Microbiology, 60*(4), 1374-1378.

Jouany, J. P., Lassalas, B., Doreau, M., & Glasser, F. (2007). Dynamic features of the rumen metabolism of linoleic acid, linolenic acid and linseed oil measured in vitro. *Lipids, 42*(4), 351-360.

Julkunen-Tiitto, R. (1985). Phenolic constituents in the leaves of northern willows: methods for the analysis of certain phenolics. *Journal of Agricultural and Food Chemistry, 33*(2), 213-217.

Kairenius, P., Toivonen, V., & Shingfield, K. J. (2011). Identification and ruminal outflow of long-chain fatty acid biohydrogenation intermediates in cows fed diets containing fish oil. *Lipids, 46*(7), 587-606.

Kaneda, T. (1977). Fatty-acids of Genu *Bacillus*-example of branched-chain preference [Review]. *Bacteriological Reviews, 41*(2), 391-418.

Kaneda, T. (1991). Iso-fatty and anteiso-fatty acids in bacteria - biosynthesis, function, and taxonomic significance. *Microbiological Reviews, 55*(2), 288-302.

Kemp, P., Lander, D. J., & Holman, R. T. (1984). The hydrogenation of the series of methylene-interrupted *cis,cis*-octadecadienoic acids by pure cultures of six rumen bacteria. *British Journal of Nutrition, 52*(1), 171-177.

Kemp, P., White, R. W., & Lander, D. J. (1975). Hydrogenation of unsaturated fatty acids by five bacterial isolates from sheep rumen, including a new species

*Journal of General Microbiology, 90*(SEP), 100-114.

Kepler, C. R., Hirons, K. P., McNeill, J. J., & Tove, S. B. (1966). Intermediates and products of biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. *Journal of Biological Chemistry, 241*(6), 1350-&.

Keweloh, H., & Heipieper, H. J. (1996). *Trans* unsaturated fatty acids in bacteria. [Review]. *Lipids, 31*(2), 129-137.

Khazaal, K., & Ørskov, E. R. (1994). The *in-vitro* gas-production technique - an investigation on its potential use with insoluble polyvinylpolypyrrolidone for the assessment of phenolics-related antinutritive factors in browse species. *Animal Feed Science and Technology, 47*(3-4), 305-320.

Khiaosa-Ard, R., Bryner, S. F., Scheeder, M. R. L., Wettstein, H.-R., Kreuzer, M., & Soliva, C. R. (2009). Evidence for the inhibition of the terminal step of ruminal α-linolenic acid biohydrogenation by condensed tannins. *Journal of Dairy Science, 92*, 177-188.

Krause, D. O., Smith, W. J. M., Brooker, J. D., & McSweeney, C. S. (2005). Tolerance mechanisms of *streptococci* to hydrolysable and condensed tannins. [Article]. *Animal Feed Science and Technology, 121*(1-2), 59-75.

Kroumova, A. B., & Wagner, G. J. (1999). Mechanisms for elongation in the biosynthesis of fatty acid components of epi-cuticular waxes. *Phytochemistry, 50*(8), 1341-1345.

Kuhnt, K., Degen, C., & Jahreis, G. (2016). Evaluation of the impact of ruminant *trans* fatty acids on human health: important aspects to consider. [Review]. *Critical Reviews in Food Science and Nutrition, 56*(12), 1964-1980.

Lee, Y. J., & Jenkins, T. C. (2011). Biohydrogenation of linolenic acid to stearic acid by the rumen microbial population yields multiple intermediate conjugated diene isomers. [Article]. *Journal of Nutrition, 141*(8), 1445-1450.

Lesschaeve, I., & Noble, A. C. (2005). Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences. *American Journal of Clinical Nutrition, 81*(1), 330s-335s.

Lim, J. N., Oh, J. J., Wang, T., Lee, J. S., Kim, S. H., Kim, Y. J., et al. (2014). *trans*-11 18:1 vaccenic acid (TVA) has a direct anti-carcinogenic effect on MCF-7 Human mammary adenocarcinoma cells. [Article]. *Nutrients, 6*(2), 627-636.

Loor, J. J., Ueda, K., Ferlay, A., Chilliard, Y., & Doreau, M. (2004). Biohydrogenation, duodenal flow and intestinal digestibility of *trans* fatty acids and conjugated linoleic acids in response to dietary forage: concentrate ratio and linseed oil in dairy cows. *Journal of Dairy Science, 87*(8), 2472-2485.

Lourenco, M., Ramos-Morales, E., & Wallace, R. J. (2010). The role of microbes in rumen lipolysis and biohydrogenation and their manipulation. *Animal, 4*(7), 1008-1023.

Lourenco, M., Van Ranst, G., Vlaeminck, B., De Smet, S., & Fievez, V. (2008). Influence of different dietary forages on the fatty acid composition of rumen digesta as well as ruminant meat and milk. *Animal Feed Science and Technology, 145*(1-4), 418-437.

Lund, M. N., Heinonen, M., Baron, C. P., & Estevez, M. (2011). Protein oxidation in muscle foods: A review. [Review]. *Molecular Nutrition & Food Research, 55*(1), 83-95.

Maia, M. R. G., Wallace, R. J., Chaudhary, L. C., Bestwick, C. S., Richardson, A. J., McKain, N., et al. (2010). Toxicity of unsaturated fatty acids to the biohydrogenating ruminal bacterium, *Butyrivibrio fibrisolvens*. *Bmc Microbiology, 10*.

Maia, M. R. G., Wallace, R. J., Chaudhary, L. C., & Figueres, L. (2007). Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 91*(4), 303-314.

Makkar, H. P. S. (2003a). Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small Ruminant Research, 49*(3), 241-256.

Makkar, H. P. S. (2003b). *Quantification of tannins in tree and shrub foliage: a laboratory manual*. Dordrecht / Boston / London: Ed. Kluwer Academic Publishers.

Makkar, H. P. S. (2005). *In vitro* gas methods for evaluation of feeds containing phytochemicals. *Animal Feed Science and Technology, 123*, 291-302.

Makkar, H. P. S., Francis, G., & Becker, K. (2007a). Bioactivity of phytochemicals in some lesser-known plants and their effects and potential applications in livestock and aquaculture production systems. *Animal, 1*(9), 1371-1391.

Makkar, H. P. S., Siddhuraju, P., & Becker, K. (2007b). *Plant secondary metabolites*. Totowa, New Jersey: Humana Press.

Mandal, G. P., Roy, A., & Patra, A. K. (2014). Effects of feeding plant additives rich in saponins and essential oils on the performance, carcass traits and conjugated linoleic acid concentrations in muscle and adipose tissues of Black Bengal goats. *Animal Feed Science and Technology, 197*, 76-84.

Mandal, G. P., Roy, A., & Patra, A. K. (2016). Effects of plant extracts rich in tannins, saponins and essential oils on rumen fermentation and conjugated linoleic acid concentrations *in vitro*. *Indian J. Anim. Health, 55*, 49-60.

Mangan, J. L. (1988). Nutritonal effects of tannins in animal feeds. *Nutrition Research Reviews, 1*, 209-231.

Marai, I. F. M., El-Darawany, A. A., Fadiel, A., & Abdel-Hafez, M. A. M. (2007). Physiological traits as affected by heat stress in sheep - A review. *Small Ruminant Research, 71*(1-3), 1-12.

Mariotti, J. P., Tomi, F., Casanova, J., Costa, J., & Bernardini, A. F. (1997). Composition of the essential oil of *Cistus ladaniferus* L. cultivated in Corsica (France). *Flavour Fragrance, 12*, 147-151.

Martin-Pinto, P., Vaquerizo, W., Penalver, F., Olaizola, J., & Oria-de-Rueda, J. A. (2006). Early effects of a wildfire on the diversity and production of fungal communities in Mediterranean vegetation types dominated by *Cistus ladanifer* and *Pinus pinaster* in Spain. [Article]. *Forest Ecology and Management, 225*(1-3), 296-305.

Martins, A. N. C., Simeonov, S. P., Frija, L. M. T., Viveiros, R., Lourenco, A., da Silva, M. S., et al. (2014). Isolation, analytical quantification and seasonal variation of labdanolic acid from the Portuguese-grown *Cistus ladaniferus*. *Industrial Crops and Products, 60*, 226-232.

Masa, C. V., Diaz, T. S., Gallego, J. C. A., & Lobon, N. C. (2016). Quantitative variation of flavonoids and diterpenes in leaves and stems of *Cistus ladanifer* L. at different ages. [Article]. *Molecules, 21*(3), 14.

McDougall, E. I. (1948). Studies on Ruminant Saliva .1. The Composition and Output of Sheeps Saliva. *Biochemical Journal, 43*(1), 99-&.

McDowell, L. R., & Valle, G. (2000). *Major minerals in forages*. Wallingford, UK: CAB International.

McIntosh, F. M., Williams, P., Losa, R., Wallace, R. J., Beever, D. A., & Newbold, C. J. (2003). Effects of essential oils on ruminal microorganisms and their protein metabolism. *Applied and Environmental Microbiology, 69*(8), 5011-5014.

McKain, N., Shingfield, K. J., & Wallace, R. J. (2010). Metabolism of conjugated linoleic acids and 18: 1 fatty acids by ruminal bacteria: products and mechanisms. *Microbiology-Sgm, 156*, 579-588.

McMahon, L. R., McAllister, T. A., Berg, B. P., Majak, W., Acharya, S. N., Popp, J. D., et al. (2000). A review of the effects of forage condensed tannins on ruminal fermentation and bloat in grazing cattle. *Canadian Journal of Plant Science, 80*(3), 469-485.

McNeill, S. H. (2014). Inclusion of red meat in healthful dietary patterns. *Meat Science, 98*(3), 452-460.

McSweeney, C. S., Palmer, B., Bunch, R., & Krause, D. O. (2001). Effect of the tropical forage calliandra on microbial protein synthesis and ecology in the rumen. *Journal of Applied Microbiology, 90*(1), 78-88.

Min, B. R., Barry, T. N., Attwood, G. T., & McNabb, W. C. (2003). The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. *Animal Feed Science and Technology, 106*(1-4), 3-19.

Minieri, S., Buccioni, A., Rapaccini, S., Pezzati, A., Benvenuti, D., Serra, A., et al. (2014). Effect of quebracho tannin extract on soybean and linseed oil biohydrogenation by solid associated bacteria: an *in vitro* study. *Italian Journal of Animal Science, 13*(3).

Miri, V. H., Ebrahimi, S. H., & Tyagi, A. K. (2015). The effect of cumin (*Cuminum cyminum*) seed extract on the inhibition of PUFA biohydrogenation in the rumen of lactating goats via changes in the activity of rumen bacteria and linoleate isomerase enzyme. *Small Ruminant Research, 125*, 56-63.

Miri, V. H., Tyagi, A. K., Ebrahimi, S. H., & Mohini, M. (2013). Plant extract enhanced ruminal CLA concentration, in vitro. *Journal of Animal and Feed Sciences, 22*(3), 219-228.

Miyagawa, E. (1982). Cellular fatty-acid and fatty aldehyde composition of rumen bacteria. [Article]. *Journal of General and Applied Microbiology*, *28*(5), 389-408.

Moon, C. D., Pacheco, D. M., Kelly, W. J., Leahy, S. C., Li, D., Kopecny, J., et al. (2008). Reclassification of *Clostridium proteoclasticum* as *Butyrivibrio proteoclasticus* comb. nov., a butyrate producing ruminal bacterium. *International Journal of Systematic and Evolutionary Microbiology, 58*, 2041-2045.

Morales-Soto, A., Oruna-Concha, M. J., Elmore, J. S., Barrajon-Catalan, E., Micol, V., Roldan, C., et al. (2015). Volatile profile of Spanish *Cistus* plants as sources of antimicrobials for industrial applications. [Article]. *Industrial Crops and Products, 74*, 425-433.

Mosley, E. E., Nudda, A., Corato, A., Rossi, E., Jenkins, T. C., & McGuire, M. A. (2006). Differential biohydrogenation and isomerization of [U-C-13]oleic and [1-C-13]oleic acids by mixed ruminal microbes. *Lipids*, *41*(5), 513-517.

Mosley, E. E., Powell, G. L., Riley, M. B., & Jenkins, T. C. (2002). Microbial biohydrogenation of oleic acid to trans isomers in vitro. *Journal of Lipid Research, 43*(2), 290-296.

Moyler, D. A., & Clery, R. A. (1997). The aromatic resins: their chemistry and uses. *SPECIAL PUBLICATION-ROYAL SOCIETY OF CHEMISTRY, 214*, 96-115.

Mueller-Harvey, I. (2006). Unravelling the conundrum of tannins in animal nutrition and health. *Journal of the Science of Food and Agriculture*, *86*(13), 2010-2037.

Nam, I. S., & Garnsworthy, P. C. (2007). Biohydrogenation of linoleic acid by rumen fungi compared with rumen bacteria. *Journal of Applied Microbiology*, *103*(3), 551-556.

Nieto, G., Diaz, P., Banon, S., & Garrido, M. D. (2010). Dietary administration of ewe diets with a distillate from rosemary leaves (*Rosmarinus officinalis L.*): Influence on lamb meat quality. *Meat Science, 84*(1), 23-29.

Ntambi, J. M., & Miyazaki, M. (2004). Regulation of stearoyl-CoA desaturases and role in metabolism. *Progress in Lipid Research, 43*(2), 91-104.

Oliveira, M. A., Alves, S. P., Santos-Silva, J., & Bessa, R. J. B. (2016). Effects of clays used as oil adsorbents in lamb diets on fatty acid composition of abomasal digesta and meat. *Animal Feed Science and Technology, 213*, 64-73.

Oyaizu, M. (1986). Studies on products of browing reactions: antioxidative activities of products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition, 44*, 307-315.

Paillard, D., McKain, N., Chaudhary, L. C., Walker, N. D., Pizette, F., Koppova, I., et al. (2007). Relation between phylogenetic position, lipid metabolism and butyrate production by different Butyrivibrio-like bacteria from the rumen. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 91*(4), 417-422.

Palmquist, D. L., & Jenkins, T. C. (2003). Challenges with fats and fatty acid methods. *Journal of Animal Science, 81*(12), 3250-3254.

Palmquist, D. L., Lock, A. L., Shingfield, K. J., & Bauman, D. E. (2005). Biosynthesis of conjugated linoleic acid in ruminants and humans. *Advances in food and nutrition research, 50*, 179-217.

Palmquist, D. L., St-Pierre, N., & McClure, K. E. (2004). Tissue fatty acid profiles can be used to quantify endogenous rumenic acid synthesis in lambs. *Journal of Nutrition, 134*(9), 2407-2414.

Papaefthimiou, D., Papanikolaou, A., Falara, V., Givanoudi, S., Kostas, S., & Kanellis, A. K. (2014). Genus *Cistus*: a model for exploring labdane-type diterpenes' biosynthesis and a natural source of high value products with biological, aromatic, and pharmacological properties. [Review]. *Frontiers in Chemistry, 2*, 19.

Patra, A. K., & Saxena, J. (2011). Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. *Journal of the Science of Food and Agriculture, 91*(1), 24-37.

Pearcy, R. W. (1978). Effect of growth temperature on fatty acid composition of leaf lipids in *Atriplex Lentiformis* (Torr.) Wats. *Plant Physiology, 61*(4), 484-486.

Piluzza, G., Sulas, L., & Bullitta, S. (2014). Tannins in forage plants and their role in animal husbandry and environmental sustainability: a review. *Grass and Forage Science, 69*, 32–48.

Piperova, L. S., Sampugna, J., Teter, B. B., Kalscheur, K. F., Yurawecz, M. P., Ku, Y., et al. (2002). Duodenal and milk trans octadecenoic acid and conjugated linoleic acid (CLA) isomers indicate that postabsorptive synthesis is the predominant source of cis-9-containing CLA in lactating dairy cows. *Journal of Nutrition, 132*(6), 1235-1241.

Polan, C. E., Tove, S. B., & McNeill, J. J. (1964). Biohydrogenation of unsaturated fatty acids by rumen bacteria. *Journal of Bacteriology, 88*(4), 1056-&.

Porter, L. J., Hrstich, L. N., & Chan, B. G. (1986). The Conversion of Procyanidins and Prodelphinidins to Cyanidin and Delphinidin. *Phytochemistry, 25*(1), 223-230.

Priolo, A., Bella, M., Lanza, A., Galofaro, V., Biondi, L., Barbagallo, D., et al. (2005). Carcass and meat quality of lambs fed fresh sulla (*Hedysarum coronarium* L.) with or without polyethylene glycol or concentrate. *Small Ruminant Research, 59*(2-3), 281-288.

Radunz, A. (1987). On the function of methyl-branched chain fatty acids in phospholipids of cell membranes of higher plants. In P. Stumpf, J. B. Mudd & W. D. Nes (Eds.), *The metabolism, structure, and function of plant lipids* (pp. 197-200). New York: Springer New York.

Raes, K., De Smet, S., & Demeyer, D. (2004). Effect of dietary fatty acids on incorporation of long chain polyunsaturated fatty acids and conjugated linoleic acid in lamb, beef and pork meat: a review. *Animal Feed Science and Technology, 113*(1-4), 199-221.

Ramalho, P. S., de Freitas, V. A. P., Macedo, A., Silva, G., & Silva, A. M. S. (1999). Volatile components of Cistus ladanifer leaves. *Flavour and Fragrance Journal, 14*(5), 300-302.

Ran-Ressler, R. R., Khailova, L., Arganbright, K. M., Adkins-Rieck, C. K., Jouni, Z. E., Koren, O., et al. (2011). Branched chain fatty acids reduce the incidence of necrotizing enterocolitis and alter gastrointestinal microbial ecology in a Neonatal Rat Model. [Article]. *Plos One, 6*(12), 10.

Rana, M. S., Tyagi, A., Hossain, S. A., & Tyagi, A. K. (2012). Effect of tanniniferous *Terminalia chebula* extract on rumen biohydrogenation, D9-desaturase activity, CLA content and fatty acid composition in *longissimus dorsi* muscle of kids. [Article]. *Meat Science, 90*(3), 558-563.

Razzaghi, A., Naserian, A. A., Valizadeh, R., Ebrahimi, S. H., Khorrami, B., Malekkhahi, M., et al. (2015). Pomegranate seed pulp, pistachio hulls, and tomato pomace as replacement of wheat bran increased milk conjugated linoleic acid concentrations without adverse

effects on ruminal fermentation and performance of Saanen dairy goats. *Animal Feed Science and Technology, 210*, 46-55.

Rincon, J., De Lucas, A., & Garcia, I. (2000). Isolation of rock rose essential oil using supercritical CO2 extraction. [Article]. *Separation Science and Technology, 35*(16), 2745-2763.

Robles, C., Bousquet-Mélou, A., Garzino, S., & Bonin, G. (2003). Comparison of essential oil composition of two varieties of *Cistus ladanifer*. *Biochemical Systematics and Ecology, 31*(3), 339-343.

Ruiz-Lopez, N., Sayanova, O., Napier, J. A., & Haslam, R. P. (2012). Metabolic engineering of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway into transgenic plants. *Journal of Experimental Botany, 63*(7), 2397-2410.

Russell, J. B. (2007). The energy spilling reactions of bacteria and other organisms. [Review]. *Journal of Molecular Microbiology and Biotechnology, 13*(1-3), 1-11.

Salem, A. Z. M., Salem, M. Z. M., El-Adawy, M. M., & Robinson, P. H. (2006). Nutritive evaluations of some browse tree foliages during the dry season: secondary compounds, feed intake and in vivo digestibility in sheep and goats. [Article]. *Animal Feed Science and Technology, 127*(3-4), 251-267.

Saminathan, M., Sieo, C. C., Gan, H. M., Ravi, S., Venkatachalam, K., Abdullah, N., et al. (2016). Modulatory effects of condensed tannin fractions of different molecular weights from a *Leucaena leucocephala* hybrid on the bovine rumen bacterial community *in vitro*. *Journal of the Science of Food and Agriculture, 96*(13), 4565-4574.

Sampath, H., & Ntambi, J. M. (2011). The role of stearoyl-CoA desaturase in obesity, insulin resistance, and inflammation. *Annals of the New York Academy of Sciences, 1243*(1), 47-53.

Sanchez-Vioque, R., Polissiou, M., Astraka, K., de los Mozos-Pascual, M., Tarantilis, P., Herraiz-Penalver, D., et al. (2013). Polyphenol composition and antioxidant and metal chelating activities of the solid residues from the essential oil industry. [Article]. *Industrial Crops and Products, 49*, 150-159.

Santos-Silva, J., Mendes, I. A., & Bessa, R. J. B. (2002). The effect of genotype, feeding system and slaughter weight on the quality of light lambs - 1. Growth, carcass composition and meat quality. *Livestock Production Science, 76*(1-2), 17-25.

Santos, E. S., Abreu, M. M., & Magalhães, M. C. F. (2016). *Cistus ladanifer* phytostabilizing soils contaminated with non-essential chemical elements. [Article]. *Ecological Engineering, 94*, 107-116.

Schmid, A., Collomb, M., Sieber, R., & Bee, G. (2006). Conjugated linoleic acid in meat and meat products: A review. *Meat Science, 73*(1), 29-41.

Scollan, N., Dannenberger, D., Nuernberg, K., Richardson, I., MacKintosh, S., Hocquette, J. F., et al. (2014). Enhancing the nutritional and health value of beef lipids and their relationship with meat quality. *Meat Science, 97*(3), 384-394.

Scollan, N., Hocquette, J. F., Nuernberg, K., Dannenberger, D., Richardson, I., & Moloney, A. (2006). Innovations in beef production systems that enhance the nutritional and health value of beef lipids and their relationship with meat quality. [Article; Proceedings Paper]. *Meat Science, 74*(1), 17-33.

Sfougaris, A. I., Nastis, A. S., & Papageorgiou, N. K. (1996). Food resources and quality for the introduced Cretan wild coat or agrimi *Capra aegagrus cretica* on Atalandi Island, Greece, and implications for ecosystem management. *Biological Conservation, 78*(3), 239-245.

Shingfield, K. J., Bernard, L., Leroux, C., & Chilliard, Y. (2010). Role of trans fatty acids in the nutritional regulation of mammary lipogenesis in ruminants. *Animal, 4*(7), 1140-1166.

Shingfield, K. J., Chilliard, Y., Toivonen, V., Kairenius, P., & Givens, D. I. (2008). Trans fatty acids and bioactive lipids in ruminant milk *Bioactive Components of Milk* (Vol. 606, pp. 3-65).

Shingfield, K. J., & Wallace, R. J. (2014). Synthesis of conjugated linoleic acid in ruminants and humans *Conjugated Linoleic Acids and Conjugated Vegetable Oils* (pp. 1-65): The Royal Society of Chemistry.

Shokryzadan, P., Rajion, M. A., Meng, G. Y., Boo, L. J., Ebrahimi, M., Royan, M., et al. (2017). Conjugated linoleic acid: A potent fatty acid linked to animal and human health. [Review]. *Critical Reviews in Food Science and Nutrition, 57*(13), 2737-2748.

Siurana, A., Ferret, A., Rodriguez, M., Vlaeminck, B., Fievez, V., & Calsamiglia, S. (2018). Strategies to modify the ruminal biohydrogenation of polyunsaturated fatty acids and the production of *trans*-10, *cis*-12 C18:2 *in vitro*. *Animal Feed Science and Technology, 235*, 158-165.

Sivakumaran, S., Molan, A. L., Meagher, L. P., Kolb, B., Foo, L. Y., Lane, G. A., et al. (2004). Variation in antimicrobial action of proanthocyanidins from *Dorycnium rectum* against rumen bacteria. *Phytochemistry, 65*(17), 2485-2497.

Skogsmyr, I., & Fagerstrom, T. (1992). The cost of antiherbivory defense - an evaluation of some ecological and physiological factors. *Oikos, 64*(3), 451-457.

Smith, A. H., Zoetendal, E., & Mackie, R. I. (2005). Bacterial mechanisms to overcome inhibitory effects of dietary tannins. *Microbial ecology, 50*(2), 197-205.

Soler, F. (2013). Intoxicaciones por plantas en animales de abasto: diagnóstico de la situación en España (Plant poisoning in animals raised for slaughter: an assessment of the situation in Spain)

*Revista de Toxicología, 30*, 27.

Sosa, T., Alias, J. C., Escudero, J. C., & Chaves, N. (2005). Interpopulational variation in the flavonoid composition of *Cistus ladanifer L.* exudate. *Biochemical Systematics and Ecology, 33*(4), 353-364.

Strumeyer, D. H., & Malin, M. J. (1975). Condensed tannins in grain-sorghum - isolation, fractionation, and characterization. *Journal of Agricultural and Food Chemistry, 23*(5), 909-914.

Sukhija, P. S., & Palmquist, D. L. (1988). Rapid Method for Determination of Total Fatty-Acid Content and Composition of Feedstuffs and Feces. *Journal of Agricultural and Food Chemistry, 36*(6), 1202-1206.

Szczechowiak, J., Szumacher-Strabel, M., El-Sherbiny, M., Pers-Kamczyc, E., Pawlak, P., & Cieslak, A. (2016). Rumen fermentation, methane concentration and fatty acid proportion in the rumen and milk of dairy cows fed condensed tannin and/or fish-soybean oils blend. [Article]. *Animal Feed Science and Technology, 216*, 93-107.

Talavera, S., Gibbs, P. E., & Herrera, J. (1993). Reproductive biology of *Cistus Ladanifer* (*Cistaceae*). *Plant Systematics and Evolution, 186*(3-4), 123-134.

Teixeira, S., Mendes, A., Alves, A., & Santos, L. (2007). Simultaneous distillation-extraction of high-value volatile compounds from *Cistus ladanifer* L. *Analytica Chimica Acta, 584*(2), 439-446.

Tolera, A., Khazaal, K., & Ørskov, E. R. (1997). Nutritive evaluation of some browse species. *Animal Feed Science and Technology, 67*(2-3), 181-195.

Tomas-Menor, L., Morales-Soto, A., Barrajon-Catalan, E., Roldan-Segura, C., Segura-Carretero, A., & Micol, V. (2013). Correlation between the antibacterial activity and the composition of extracts derived from various Spanish Cistus species. *Food and Chemical Toxicology, 55*, 313-322.

Toral, P. G., Chilliard, Y., & Bernard, L. (2012). Short communication: In vivo deposition of [1-C-13]vaccenic acid and the product of its Delta(9)-desaturation, [1-C-13]rumenic acid, in the body tissues of lactating goats fed oils. *Journal of Dairy Science, 95*(11), 6755-6759.

Troegeler-Meynadier, A., Palagiano, C., & Enjalbert, F. (2014). Effects of pH and fermentative substrate on ruminal metabolism of fatty acids during short-term *in vitro* incubation. [Article]. *Journal of Animal Physiology and Animal Nutrition, 98*(4), 704-713.

Tsydendambaev, V. D., Christie, W. W., Brechany, E. Y., & Vereshchagin, A. G. (2004). Identification of unusual fatty acids of four alpine plant species from the Pamirs. *Phytochemistry, 65*(19), 2695-2703.

Van Soest, P. J. (1994). *Nutritional Ecology of the Ruminant* (2nd ed.). Ithaca, NY: Cornell University Press.

Van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *Journal of Dairy Science, 74*(10), 3583-3597.

Vasta, V., Aouadi, D., Brogna, D. M. R., Scerra, M., Luciano, G., Priolo, A., et al. (2013). Effect of the dietary supplementation of essential oils from rosemary and artemisia on muscle fatty acids and volatile compound profiles in Barbarine lambs. *Meat Science, 95*(2), 235-241.

Vasta, V., & Bessa, R. J. B. (2012). Manipulating ruminal biohydrogenation by the use of plants bioactive compounds. In A. K. Patra (Ed.), *Dietary Phytochemicals and Microbes* (pp. 263-284). Netherlands: Springer.

Vasta, V., Jeronimo, E., Brogna, D. M. R., Dentinho, M. T. P., Biondi, L., Santos-Silva, J., et al. (2010a). The effect of grape seed extract or *Cistus ladanifer L.* on muscle volatile compounds of lambs fed dehydrated lucerne supplemented with oil. *Food Chemistry, 119*(4), 1339-1345.

Vasta, V., & Luciano, G. (2011). The effects of dietary consumption of plants secondary compounds on small ruminants' products quality. *Small Ruminant Research, 101*(1-3), 150-159.

Vasta, V., Makkar, H. P. S., Mele, M., & Priolo, A. (2009a). Ruminal biohydrogenation as affected by tannins *in vitro*. *British Journal of Nutrition, 102*(1), 82-92.

Vasta, V., Mele, M., Serra, A., Scerra, M., Luciano, G., Lanza, G., et al. (2009b). Metabolic fate of fatty acids involved in ruminal biohydrogenation in sheep fed concentrate or herbage with or without tannins. *Journal of Animal Science, 87*, 2674-2684.

Vasta, V., Nudda, A., Cannas, A., Lanza, M., & Priolo, A. (2008). Alternative feed resources and small ruminants meat and milk quality. A review. *Animal Feed Science and Technology, 147*, 223-246.

Vasta, V., Pennisi, P., Lanza, M., Barbagallo, D., Bella, M., & Priolo, A. (2007). Intramuscular fatty acid composition of lambs given a tanniniferous diet with or without poluethylene glycol supplementation. *Meat Science, 76*, 739-745.

Vasta, V., Priolo, A., Scerra, M., Hallett, K. G., Wood, J. D., & Doran, O. (2009c). Delta(9) desaturase protein expression and fatty acid composition of longissimus dorsi muscle in lambs fed green herbage or concentrate with or without added tannins. *Meat Science, 82*(3), 357-364.

Vasta, V., Yáñez-Ruiz, D. R., Mele, M., Serra, A., Luciano, G., Lanza, G., et al. (2010b). Bacterial and protozoa communities and fatty acid profile in the rumen of sheep fed a diet containing added tannins. *Applied and Environmental Microbiology, 76*, 2549-2555.

Verdeguer, M., Blazquez, M. A., & Boira, H. (2012). Chemical composition and herbicidal activity of the essential oil from a Cistus ladanifer L. population from Spain. *Natural Product Research, 26*(17), 1602-1609.

Vieira, M., Bessa, L. J., Martins, M. R., Arantes, S., Teixeira, A. P. S., Mendes, A., et al. (2017). Chemical composition, antibacterial, antibiofilm and synergistic properties of essential oils from *Eucalyptus globulus* LABILL. and seven Mediterranean aromatic plants. [Article]. *Chemistry & Biodiversity, 14*(6), 12.

Viuda-Martos, M., Sendra, E., Perez-Alvarez, J. A., Fernandez-Lopez, J., Amensour, M., & Abrini, J. (2011). Identification of flavonoid content and chemical composition of the essential oils of Moroccan herbs: myrtle (*Myrtus communis* L.), rockrose (*Cistus*

*ladanifer* L.) and Montpellier *cistus* (*Cistus monspeliensis* L.). *Journal of Essential Oil Research, 23*(2), 1-9.

Vlaeminck, B., Fievez, V., Cabrita, A. R. J., Fonseca, A. J. M., & Dewhurst, R. J. (2006). Factors affecting odd- and branched-chain fatty acids in milk: A review. *Animal Feed Science and Technology, 131*(3-4), 389-417.

Waghorn, G. (2008). Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production-Progress and challenges. *Animal Feed Science and Technology, 147*(1-3), 116-139.

Wallace, R. J., Chaudhary, L. C., McKain, N., McEwan, N. R., Richardson, A. J., Vercoe, P. E., et al. (2006). *Clostridium proteoclasticum*: a ruminal bacterium that forms stearic acid from linoleic acid. *Fems Microbiology Letters, 265*(2), 195-201.

Wang, Y., Jacome-Sosa, M. M., & Proctor, S. D. (2012). The role of ruminant *trans* fat as a potential nutraceutical in the prevention of cardiovascular disease. [Review]. *Food Research International, 46*(2), 460-468.

Wasowska, I., Maia, M. R. G., Niedzwiedzka, K. M., Czauderna, M., Ribeiro, J., Devillard, E., et al. (2006). Influence of fish oil on ruminal biohydrogenation of C18 unsaturated fatty acids. *British Journal of Nutrition, 95*(6), 1199-1211.

Weyerstahl, P., Marschall, H., Weirauch, M., Thefeld, K., & Surburg, H. (1998). Constituents of commercial Labdanum oil. *Flavour and Fragrance Journal, 13*(5), 295-318.

Williams, A. G., & Coleman, G. S. (1992). *The rumen protozoa*. New York: Springer-Verlag.

Wongtangtintharn, S., Oku, H., Iwasaki, H., & Toda, T. (2004). Effect of branched-chain fatty acids on fatty acid biosynthesis of human breast cancer cells. [Article]. *Journal of Nutritional Science and Vitaminology, 50*(2), 137-143.

Wood, J. D., Enser, M., Fisher, A. V., Nute, G. R., Sheard, P. R., Richardson, R. I., et al. (2008). Fat deposition, fatty acid composition and meat quality: A review. *Meat Science, 78*(4), 343-358.

Wyness, L. (2016). The role of red meat in the diet: nutrition and health benefits. [Article; Proceedings Paper]. *Proceedings of the Nutrition Society, 75*(3), 227-232.

Yeruham, I., Orgad, U., Avidar, Y., Perl, S., Liberboim, M., Adler, H., et al. (2002). A urinary retention syndrome in beef cows probably caused by ingestion of *Cistus salvifolius*. *Revue De Medecine Veterinaire, 153*(10), 627-632.

Zidane, H., Elmiz, M., Aouinti, F., Tahani, A., Wathelet, J., Sindic, M., et al. (2013). Chemical composition and antioxidant activity of essential oil, various organic extracts of *Cistus ladanifer* and *Cistus libanotis* growing in Eastern Morocco. *African Journal of Biotechnology*, *12*(34).